Experimental studies on the spontaneous entrapment of macromolecules inside liposomes: synthetic models of minimal cells

Dissertation

zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.)

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Master of Science in Molekularbiologie (M.Sc.)

geboren am 25.06.1980 in Rom, Italien.

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Presented to the Council of the Faculty of Biology and Pharmacy of

the Friedrich-Schiller-University Jena

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1 Introduction

"Everything should be as simple as it is, but not simpler."
Albert Einstein

Biology literally means the study of Life and it should not surprise if such discipline has ancient roots, in fact, human beings were always curious to know the secrets and the rules that govern the Nature. During the course of history, they tried to give answers to the existential questions like "what is life?" "Where do we come from?" "How did life arise?" We have not found the answers yet, but a relatively young research field, the synthetic biology, has made progress in this matter. When we look at modern cells, we have to admit that even the simplest one is composed by thousands elements connected each other by a complicated and intriguing metabolic network. This complexity is due to billions of years of evolution and it is reasonable thinking that the protocells were not as complex as the actual ones. We do not try to reproduce an artificial full-fledged cell, but instead we would reproduce those simple complexes that brought to the first cell. The approach that we consider here does not involve genetic manipulation, but it is a chemical approach; we focus on the preparation of semi-synthetic cells via encapsulation of biochemicals inside lipid vesicles. In the next paragraphs, we introduce a brief account on the scientific explanation of the origin of life to give a better view on the framework of our research on the primitive forms of metabolism.

1.1 Scientific explanation of the origin of life

Aristoteles (384 B.C. – 322 B.C.) on the base of the earlier idea of Empedocles, thought that life has spontaneously arisen from inanimate matter, giving the birth to the abiogenesis theory. From this moment, and for several centuries, philosophers and intellectuals generally accepted the idea of spontaneous generation until the birth of the experimental scientific approach.

During the XVII century, many scientists started to perform experiments to see if organisms and microorganisms could be indeed generated from inanimate matter. The debate persisted for about two hundred years ending in 1862 with the brilliant experiments executed by Luis Pasteur, showing the impossibility to make living beings from non-living matter.

The XIX century is considered as starting point of the modern biology even for the innovative theory of evolution formulated by Charles Darwin in his famous book *The Origin of Species* published in 1859. Darwin proposed that all the present organisms have

originated from few or even one common ancestor. He claimed that a fundamental role was played by the natural selection in order to make new kinds of living forms and the given variation is heritable. The naturalist author did not try to give an answer to the origin of life, but he figured out a scenario in which life could have arisen, he imagined a “warm pond” where the right ingredients were present (namely the famous primordial soup).

At the beginning of the 20th century, Aleksandr Oparin and John Haldane resumed the theory of the spontaneous generation moving it on a larger scale of years and from a very new point of view (Oparin 1924, 1953 and 1957; Haldane 1929, 1954, 1967). They assumed that in primitive times there should be no free oxygen, since it makes some compounds needed for evolution of life, e.g. sugars and amino acids, unstable. They imagined the primeval soup surrounded by a reducing atmosphere in which the first molecules formed. According to the Oparin’s theory, there was not a gap between the inorganic matter and living cells, but starting from simple atoms and small molecules like water, nitrogen and carbonic dioxide there was a spontaneous and extremely slow increase of molecular complexity, giving simple organic molecules first (e.g. amino acids, nucleosides, nucleotide and small lipids). Then, thanks to the only action of basic laws of physics and chemistry, they could react to give complex molecules, which in turn assembled into simple functional complexes and then into the cells. There has been a kind a flux of reactions that brought from inanimate material present on the Earth to the first forms of life (figure 1.1), we can say that the matter underwent to a real “chemical evolution”. This theory is called “the continuity principle” (Oparin, 1924; De Duve, 1991; Morowitz, 1992) and the scientific community still take it for granted. Although it is certainly true that starting from a certain moment “life comes from life” (Pasteur experiments), the very first living organism, probably a tiny primitive and not very efficient microorganism, necessarily originated from non-living matter (abiogenesis). Note, that the so-called “panspermia” theory (Hoyle and Wickramasinghe, 1999; Britt, 2000; Shostak, 2003) – namely, the not-fully convincing idea that life arrived to the Earth in form of spores from the space – just shifts the problem from abiogenesis on Earth on abiogenesis on another planet.

From this perspective, then, the mechanisms underlying abiogenesis becomes the central topic of the old question: how did life originate on Earth? As mentioned, it seems reasonable thinking that the first organisms were unicellular cells, not so complex like the ones that we know, and that the path that brought to life, even if it is still largely unknown, was characterized by combinatorial trials at the molecular and supramolecular level. In such scenario, inefficient or unreactive systems were just discarded, whereas slowly evolving chemical systems, which eventually lead to the formation of cell structures with minimal

complexity, were selected. Such systems, although very simple and rudimentary, were able to perform simple functions and gave rise to early life on our planet.

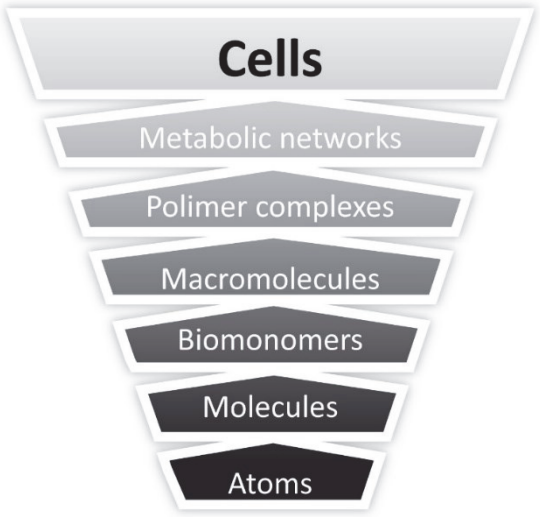

Figure 1.1. Arbitrary scale to explain the continuity principle.

It is intriguing and at the same time rewarding, trying to imagine how these *minimal cells* look like, and also making them in the laboratory (Luisi and Varela, 1990). This practice will allow us to understand, in a stepwise manner, what could have happened billions of years ago. The concepts and the experimental implementation “minimal cell” will be discussed more in detail in the paragraph 1.4.

1.2 Prebiotic chemistry

The new view about the origin of life originated by the Oparin-Haldane theory unlocked the door to series of experiments (some more brilliant, other less) directed to reproduce some basic biological compounds in prebiotic conditions. It might be that making life from non-life was a titanic effort, because primeval Earth, billions of years ago, was completely different from the Earth that we well know now, and only in those particular conditions, life could arise. It is quite logical that understanding, as much as possible, the condition on the Earth crust at the earliest stages could help to explain the origin of the first forms of life. Our planet reached its actual mass around 4.5 billions of years ago; it had a melted metal core and a particular atmosphere (Halliday, 2000). After its birth, celestial bodies bombarded the Earth, causing the formation of a too warm and dense atmosphere to permit the emergence of life. The most important element for life is obviously water, it could have

formed after the cooling of the crust, the analysis of some zircon crystals showed that it happened 4.4 billions of years ago (Wilde et al. , 2001; Mojzsis et al., 2001).

Microfossils of prokaryotes, the simplest organisms on our planet, were found in Australia and they are dated 3.5 billions of years ago -"only" some millions of years after the formation of the oceans (Schopf, 1992, 1993, 1998), so all the scientists agree that life started between 3.5 and 3.9 billions of years ago. There was a long disputation, as already mentioned before, on the composition of the atmosphere at that time, for one discussed theory it was reducing (CH_4 + N_2, NH_3 + H_2O or CO_2 + H_2 + N_2), for another it was neutral (CO_2 + N_2 + H_2O), but for both there was no free oxygen (Lazcano and Miller, 1996).

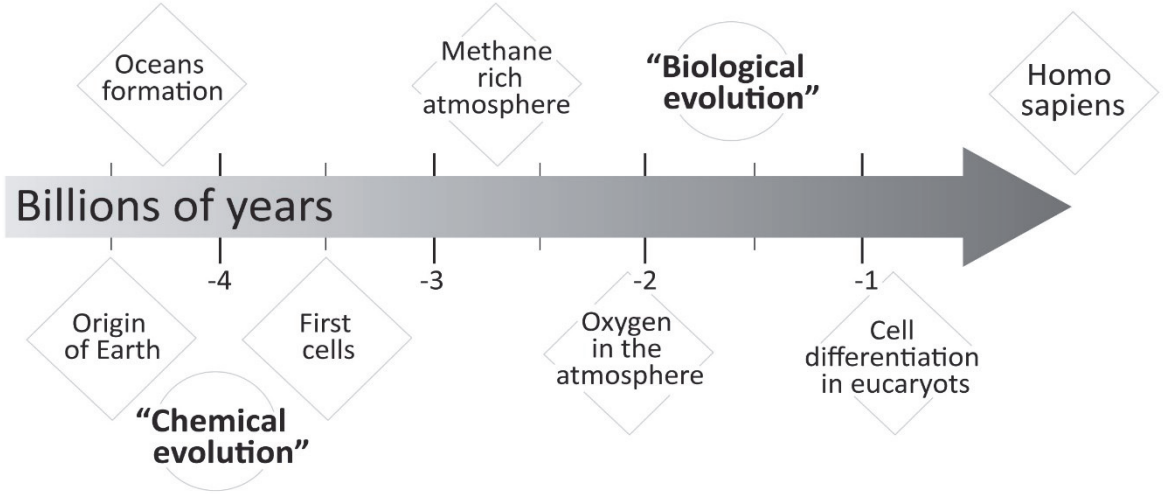

Figure 1.2. Scheme showing some stages that characterized the Earth from its origin, passing through the formation of the first cells arriving to the emergence of the *Homo sapiens*.

It is assumed that some chemical processes can be made in laboratory in order to reproduce the transition to life, once we find the right combination of prebiotic compounds and the right conditions. Some theorists have suggested that in such reducing atmosphere, the electrical energy due to the lighting could have catalysed the synthesis of some small molecules of life, like amino acids and fatty acids.

This theory become stronger in 1953 by the remarkable experiment performed by Stanley Miller when he was a PhD student of the famous Harold C. Urey. Miller wanted to verify if organic molecules could be obtained by spontaneous generation reproducing primordial atmosphere.

This is a historical experiment, he reproduced the atmosphere with hydrogen, water, methane and ammonia in a closed chamber and he used the electricity to simulate the lighting discharge. In another flask there was boiling water in order to mimic the warm oceans. After some weeks, the analysis of the content in the collecting flask showed the presence of organic components, the most impressing ones were some amino acids (e.g. glycine, alanine, glutamic acids), which are fundamental building blocks of proteins, the

most important molecules for life (Miller,1953). It is now believed that the atmosphere was not reducing, but it does not mean that this experiment loses its relevance.

After this experiment, many other scientists tried to reproduce fundamental prebiotic compounds and it is worth mentioning Joan Orò. He actually wanted to reproduce the Miller's experiment, but using an aqueous solution containing hydrogen cyanide (HCN) and ammonia (NH_3). What he obtained from this experiment was terrific, in addition to some amino acids there was an amazing amount of adenine (Orò, 1960), that has a big significance in biology because it is present in nucleic acids and in adenosine triphosphate (ATP). These successful results made the scientific community optimistic at that time, but we are still far from answer to the question about the origin of life. In fact, amino acids and adenine are bio monomers, so how could they form bio oligomers or biopolymers? The question gets more complicated if we go through the modern biology and we try to understand how the genetic code came out or if we think about the first metabolism. It is not obvious that one day, eventually we could fill these gaps, because these events have happened billions of years ago, we have no evidences or fossils to study and the conditions are difficult to verify and even to imagine.

1.3 Autopoiesis and Cellular Life

When we look at the modern cells, even the simplest ones like prokaryotes, we have to admit that these are complex systems, made by a huge number of components like genes, proteins and with different metabolic paths. We cannot imagine that the first cells had such a sophisticated organisation, but more probably, they arose from the warm pond as "limping" cells (Luisi et al., 2006). All the biologists agree that the evolution has started from LUCA (last universal common ancestor) that was already a family of cells containing their own genome, capable to synthetize proteins and self-replicate. Before cells reached that level, there should have been a certain period of time in which the chemical evolution was characterized by trials and in which those "limping cells" were simple and capable only of some of the features and owing only some of the structures that distinguish the modern cells. Before trying to make an artificial cell, we have to define what cellular life is. It could seem an easy deal, but in the past, it was subject of debate for long time. In the Seventies of the last Century two scientists, Humberto R. Maturana and his student Francisco R. Varela, introduced the notion of autopoiesis (from Greek *auto* = self, *poiesis* = production) (Varela et al., 1974; Maturana and Varela, 1980). They gave three properties to living systems: self-maintenance, self-reproduction and capability to evolve. The semi-permeable boundary plays a central role in the autopoiesis theory. It is thanks to this barrier that nutrients are taken up from the external medium and waste products are discarded, in order

to maintain the cell identity (homeostasis). The cell, as an autopoietic unit, is an open system delimited by a membrane and within there is a network of reactions adapted to guarantee its conservation and to produce the boundary components, keeping a link between the cell itself and the external environment, this link is the metabolism. The cyclic logic of cellular life is represented in Figure 1.3. A minimal autopoietic system consists in a self-bounded system able to uptake a precursor **P** from the external medium and to transform it into the boundary elements **S**, which can undergo a degradation process to **W**. The relative rates of these processes determine if the system can grow, stay in homeostatic state or die (Stano and Luisi, 2010).

The concept of autopoiesis is connected to the so called “compartment approach”, a theory that bases the origin of life on the formation of a spherical and closed boundary. The logic of this idea is that all the living being are composed by cells or they are a cell and the interaction with the external medium is due to the boundary. We have also to point out that cell-like compartments can be formed spontaneously by prebiotic molecules like fatty acids (Deamer, 1985; 1998; Bachmann et al., 1992; Walde et al., 1994).

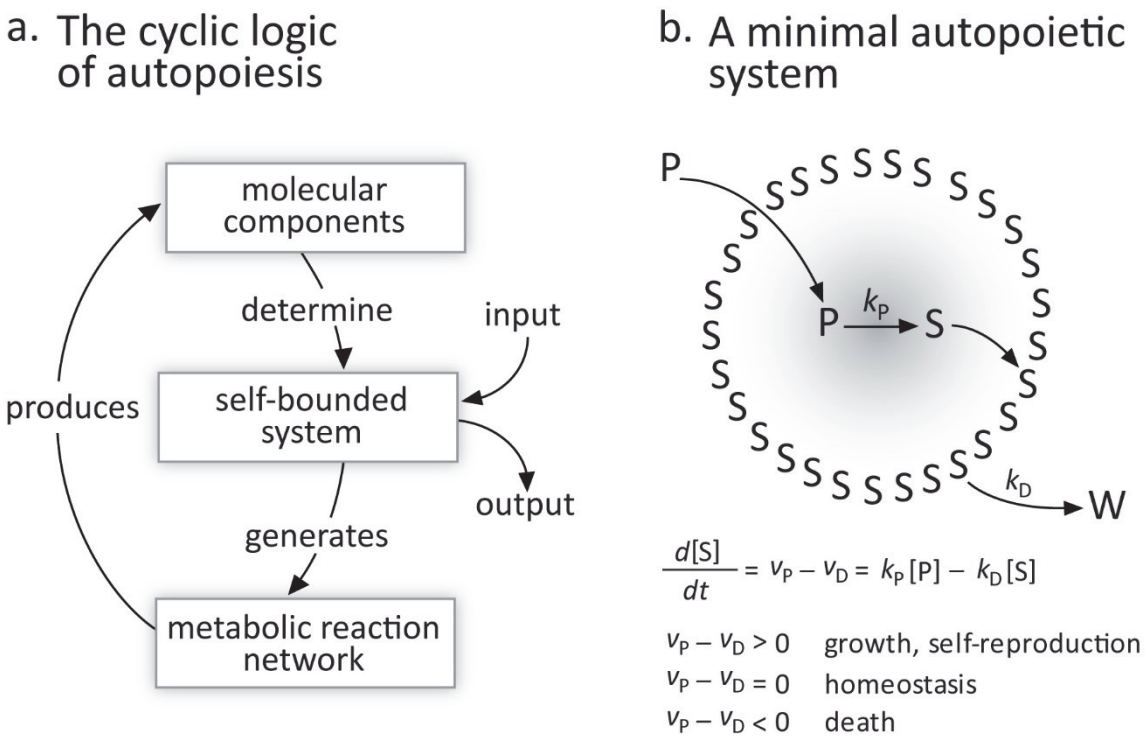

Figure 1.3. Autopoiesis. (a)The cyclic logic of cellular life. The cell, a self- bounded system, generates a metabolic reaction network that produces molecular components that determine… and so on. (b) A minimal autopoietic system characterized by two competitive reactions, one to build and another one to destroy the boundary components. (Picture redrawn from Stano and Luisi, 2010)

The current trend is studying the potential functional activities of “minimal” systems, which could form without the action of any catalysts, for example, we can study how vesicles can entrap some molecules while they are spontaneously forming in solution and triggering a simple metabolism. The idea is that the first biopolymers could be generated, by chance, thanks to spontaneous reactions driven by thermodynamic laws. Then they started to work together to make biochemical systems, which were complex, organized and probably compartmentalized, to arrive to a population of protocells capable to make simple functions, even if with lower efficiency, like nucleic acids replication or the synthesis of small proteins or lipids.

1.4 The *minimal cell* project

> “In nature we never see anything isolated, but everything in connection with something else which is before it, beside it, under it and over it”.
> **Johann Wolfgang von Goethe**

With the *minimal cell* project all the last theories are, in a way, summed up. The starting point is the consciousness that the first cells were much simpler than the actual ones, so the aim of this project is not to make a modern cell, but instead to make simple models that mimic some of the huge number of reactions and structures that characterize this life unit. We can define a minimal cell as a system containing the minimum and sufficient number of components to be “alive” and it has to be able of self-maintenance (metabolism), reproduction, and evolvability (Luisi et al., 2006). Evolvability is actually a Darwinian notion and it is more inherent to a population than to a cell. From this point of view, it should be better consider a family of *minimal cells* subject to the environment and genetic evolution. Is it possible to imagine a cell like that and to assemble it in the lab?
From the operative viewpoint, we can say that it is possible encapsulating different components inside vesicles, but what kind of molecules can we use to make such cell?
There are mainly two kinds of approaches called respectively top-down and bottom-up.
With the bottom-up approach scientists try to reproduce *minimal cells* starting from simple prebiotic molecules, examples are the experiments of Miller and Orò already described in paragraph 1.2, and the more recent work of Sutherland’s group showing the biosynthesis of mononucleotides (Powner et al., 2009). According to the observation made by Eschenmoser and Kisakürek (1996) this approach can help to understand better some possible chemical steps, which brought life on the Earth, even if it cannot describe and verify the exact path. A *minimal cell* composed by plausible prebiotic components should be able to auto-maintain itself absorbing compounds from the outside medium and transforming them in the constituents of the protocell, so it should be an autopoietic unit.

This kind of approach has some theoretical and physical limits, for example, we do not know a biochemical compound that is able to auto-replicate and to make some catalytic functions like forming membrane lipids. A model was proposed, which uses ribozymes, molecules that theoretically can supply the features anticipated before.

This model was described in 2001 by our collaborator Pier Luigi Luisi with Jack Szostak and David Bartell. It is a protocell composed by two ribozymes inside a vesicle (Szostak et al., 2001). The concept is showed in Figure 1.4, a lipid vesicle containing two ribozymes, one (Rib2) is able to catalyse the synthesis of the membrane components; the other one (Rib1) is a RNA-replicase that can replicate itself and Rib2. In this way, only with two ribozymes, we have a minimal protocell able to reproduce itself. While Rib1 synthetises the components inside the cell, the second ribozyme produces the lipids, increasing the cellular surface and allowing cellular division in other two cells and this theory is supported by the experiments made at the beginning of Nineties in Zurich in Luisi's group (Walde et al.,1994; Bachmann et al., 1992; Luisi, et al, 1993).

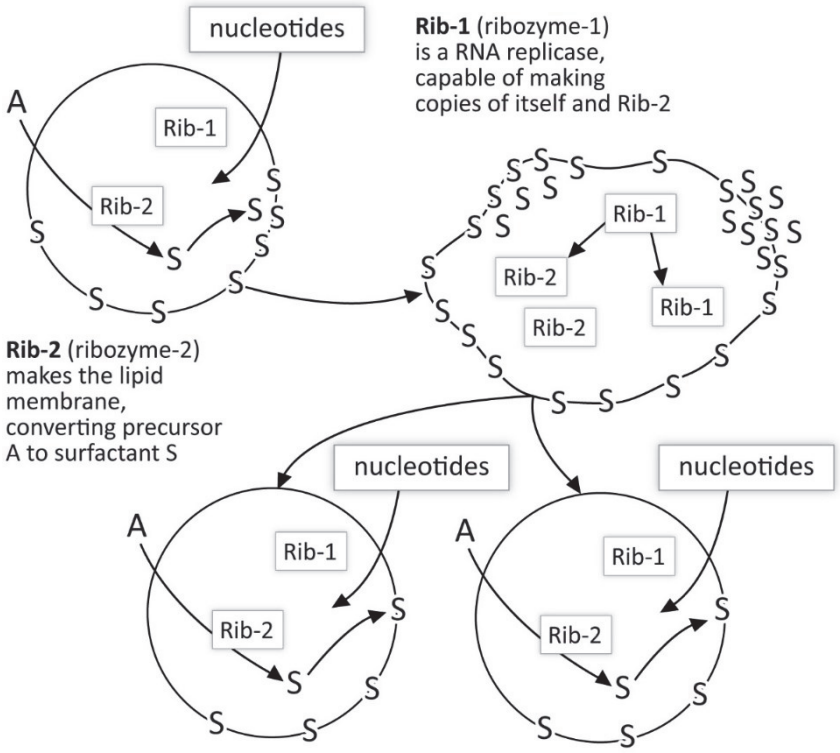

Figure 1.4. RNA *minimal cell* (redrawn from Szostak et al., 2001)

The "ribocell", however, is still a non-concrete example of protocell, in the sense that it cannot be constructed in the laboratory as the two ribozymes that would be needed have not been discovered yet. However, the ribocell is anyway interesting from a theoretical point of view. So, how can we proceed experimentally?

Other scientists considered the second approach, the so-called “top-down”, where the starting point here is the modern cell. Ideally, a *minimal cell* is obtained by reducing the complexity of modern biological cells in order to obtain a simplified one. The genomes of the simplest micro-organisms were analysed and compared, to see the basic set of genes for a minimal form of life. The “minimal genome” is defined as the minimal number of (prokaryotic) genes needed to make the cell functional even with a low efficiency, without repair mechanisms or regulation. Obviously, we have to distinguish between the genome of parasites and free-living pathogens, the first ones could have smaller genome than the latter ones, but it is due only for the symbiosis with a hosting body, which supplies the lacking materials. The minimal genome was studied by various groups like Mushegian, Koonin and recently by the group of Moya. The latter claimed that 206 genes are enough for a minimal cell, as showed in Table 1.1 (Gil et al., 2004).

At the first sight, this number could appear small, especially if we compare it with the genome of *Mycoplasma genitalium*, the microorganism with the smallest number of genes, namely 483. However, even 206 genes can be too many for early cells formed billions of years ago. Is the synthesis of such a long DNA plausible on the primeval Earth? Very probably, when we talk about protocells we have to reduce drastically the number of genes involved.

Table1.1. Minimal genome proposed by Moya and collaborators. (Gil et al., 2004).

DNA metabolism	**16**
Basic replication machinery	13
DNA repair, restriction and modification	3
RNA metabolism	**106**
Basic transcription machinery	8
Translation: aminoacyl-tRNA synthesis	21
Translation: tRNA maturation and modification	6
Translation: ribosomal proteins	50
Translation: ribosome function, maturation and modification	7
Translation factors	12
RNA degradation	2
Protein processing, folding and secretion	**15**
Protein posttranslational modification	2
Protein folding	5
Protein translocation and secretion	5
Protein turnover	3
Cellular processes	**5**
Energetic and intermediary metabolism	**56**
Poorly characterized	**8**
TOTAL	**206**

We can speculate that the first enzymes were not as sophisticated as the actual ones and also less in number, for example we can imagine that at that time only one polymerase could be enough to synthetize DNA and RNA. Moreover, the first ribosomes were probably simpler and not composed by all the 55 proteins that form the modern prokaryotic ribosome. From this point of view, we can imagine that the primitive genome was probably poorer (Luisi et al., 2002).

In the context of synthetic biology it is remarkable the work made by the group of Craig Venter, who synthesized a minimal set of genes and insert it into a modified *Mycoplasma mycoides*, leading to a self-reproducing bacteria (Gibson et al., 2010). However, it should be pointed out that such an approach was possible only thanks to the built-in metabolic machinery present in the bacterium, which acted as "chassis" where the synthetic genome was "plugged-in".

The research group where I made my PhD work and the collaborators in Rome, in particular the EU-FP6 Synthcell, aim at the construction of *minimal cells* from a still different direction, which is intermediate to bottom-up (prebiotic-like) and top-down (à la Venter), in the sense that uses modern molecules but it follows a bottom-up (constructive) approach. As already mentioned before, our methodology does not involve genetic manipulation, instead we chose a chemical approach, as part of the semi-synthetic biology. Our goal is to construct a minimal cell using the physical and chemical forces that drive molecules to auto-assemble, for example, we use the property of single lipid molecules to make vesicles when they are in aqueous solution, entrapping modern biomolecules as nucleic acids or enzymes and follow the reactions inside those compartments. We can say that it is a revisited bottom-up approach, aiming to understand the possible events that could link the origin of life phenomenon to the spontaneous organization of the matter into cellular structures. In Figure 1.5 it is shown a simplification of a semi-synthetic minimal cell (SSMC) and the method used in our research group.

In the Eighties, the chemical implementation of autopoiesis theory was developed with a system composed by reverse micelles, aqueous micelles and lipid vesicles. All these systems were able to grow and reproduce, showing a potential duplicating mechanism of protocells (Luisi and Varela, 1990; Stano and Luisi, 2010).

During the last decades, several reactions have been realized inside lipid or fatty acid vesicles, as the enzyme polymerization of ADP in poly (A) using polynucleotide phosphorylase (Walde et al., 1994); then the RNA replication through Qβ replicase (Oberholzer et al., 1995a; Kita et al., 2008) and a polymerase chain reaction (Oberholzer et al., 1995b). The Nineties were characterized by many pioneer discoveries as the first protein expression driven by ribosomes in liposomes (Oberholzer et al., 1999) to arrive at

the beginning of Two thousand with the synthesis of GFP, Green Fluorescent Protein (Yu et al., 2001).

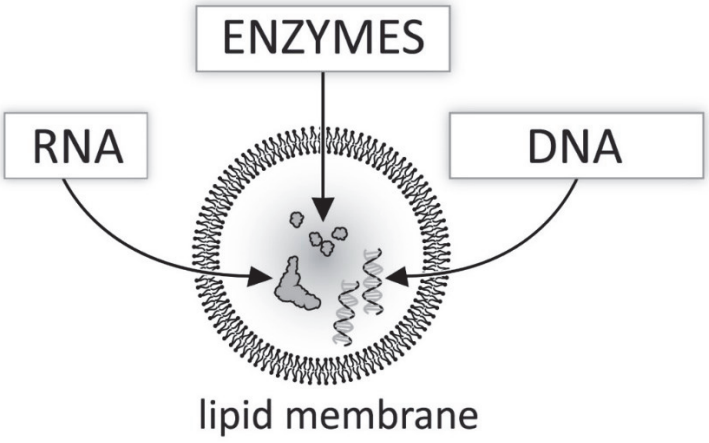

Figure 1.5 Semi-synthetic minimal cell. (Redrawn after Luisi et al., 2006)

1.5 Lipid vesicles as model of primitive cells

The research discussed in previous paragraphs suggests that an important constituent for a cellular life is the membrane. It is a layer surrounding the cell and it has different functions, it distinguishes the inner of the cell from the external medium and at the same time, it is a link between them. Indeed, it has a critical role in discriminating molecules allowed to pass through it, going into the internal cavity or discarding outside. It has also the role to protect the nucleic acids (prokaryotes have no nucleic membrane) and other organelles. We can say that the formation of the first membranes had a terrific importance in the path that brought the matter from the non-living to living state. The modern membrane is composed by different molecules, the majority are phospholipids arranged in a double layer in which are present proteins and other kinds of lipids with different structures and functions (for example proteins forming ionic channels).

In the past, it was claimed that the first cells could have been formed on clay or mineral surfaces, but it is quite difficult that all the biomolecules could organize on a solid surface, so more probably the first stages took place in water, as assumed by Darwin.

The research field of semi-synthetic biology tries to make simple artificial models of natural structures, obviously the primitive membranes were not as organised and complex as the modern ones and were probably formed by fatty acids of different lengths without ionic channels, such sophisticated structures are the product of evolution, even if some years ago was demonstrated that α-hemolysin could mimic these structure procuring pores in the membrane (Noireaux at al., 2003).

It was found that lipid vesicles were the best option to make membrane models, the most well known nano- or micro-metric compartments used for these type of experiments are

water-in-oil emulsions (Torre et al., 2014; Pietrini and Luisi, 2004), inverse micelles (Bachmann et al.,1990), liposomes (Chen et al., 2004; Soga et al.,2014) and giant-vesicles (Kurihara et al., 2011). In this thesis, we focus on liposomes or phospholipids vesicles (double-layer lipid vesicles).

Lipid vesicles (from Latin *vesicular, -ae*, small bladder) are closed compartments able to form spontaneously in aqueous solution and in which one or more lipid bilayers separate the inner from the bulk exactly like the cell membranes do and in their pool all the reactions are realized. Other similarity with the natural cell bilayer is the composition, the amphipathic molecules, which have the important feature for the origin of life to aggregate spontaneously thanks only to thermodynamic forces (Alberts, 2008). The amphipathic molecules are organised in a head-tail geometry, a hydrophilic head and one or two hydrophobic tails, the first part is in contact with the water inside and outside the vesicle and the tails interact each other avoiding water.

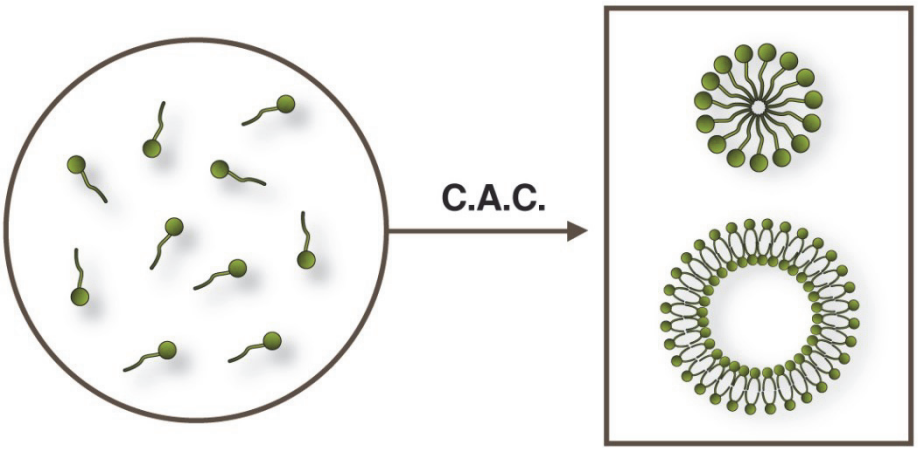

Figure 1.6 Formation of micelles or liposomes starting from amphipathic molecules.

The critical aggregation concentration (c.a.c., minimal surfactant concentration necessary to form the aggregate) influences the vesicles formation as well as many other factors like the pH, the temperature, the nature of the solvent etc.

Liposomes present some limits, for example their low permeability to molecules and the impossibility to let them fuse between each other, bringing to the difficulty to exchange material or the encapsulation of solutes.

From the other hand we have seen in the past that liposomes can proliferate when surfactant molecules are added in solution, this phenomenon is called “matrix effect” (Bloechliger et al., 1998). Moreover, they are not at thermodynamic equilibrium, but more

in a kinetically trapped state. The energy barrier is high and this equilibrium is not easy to reach, so vesicles, once formed, are kinetically stable for hours or days.
It is also possible entrap molecules inside liposomes, in fact they are extensively used as drug delivery in bio-medicine and depending on the chemical nature of the drugs they can be in the aqueous pool or on the membrane surface.

1.6 Aim of the study

LUCA was defined as a family of cells, they were already compartmentalized structures containing their own DNA, RNA and proteins separated and protected from the external environment by the lipid membrane. Even if they were present on the Earth as single cells, they had developed a significant complexity due to the presence of a genome and thousands components connected and sustained by a labyrinth of metabolic pathways. We have already said that this complexity was drastically lower in the early stages of chemical evolution, namely during the organization of the matter into the first quasi-living organisms. The path that brought to life is corroborated by several gaps that we have not filled yet. One of the crucial points in this research field is the development of the first metabolism; in particular, the main question from which all these studies came out is: did the metabolism start in a diluted solution, as suggested by Darwin, or inside compartments?
Both the scenarios are difficult to imagine, because if on one hand there is the problem of the simultaneous co-entrapment of a big number of solute, on the other hand there is the fact that the membrane is not permeable to macromolecules.
The starting point of my project are two studies made in my group, in which it was showed that, within a population of liposomes with radius of about 100 nm, some liposomes were capable of protein expression (cell-free synthesis of the fluorescent protein GFP), and surprisingly the average yield of fluorescent protein in those “special” liposomes was six times higher than in bulk. Such an observation was unexpected because, according to the Poisson distribution, the probability to co-entrap all solutes (about 80) required for cell-free protein synthesis was approximatively zero. Therefore, it was made the hypothesis that solutes were encapsulated as if their local concentration was twenty times higher than the expected one (de Souza et al., 2009), being these conditions met only for a small number of liposomes. The second paper reported a work focused to testing this initial hypothesis. In particular, a model protein – ferritin – was employed. Ferritin was used because it is a large protein, containing several thousands iron atoms and it can be detected using an electron microscope. Actually, ferritin is a classical probe for electron microscopy. After counting the number of ferritin molecules inside about eight thousand liposomes, the study showed that 90% of vesicles were empty, so they did not entrap any molecule of solute, others were filled with a small number of ferritin and very few vesicles (less than 1%),

contained a large number of ferritin, so that the ferritin concentration in the lumen of these liposomes become very high, up to about 10-20 times more concentrated than the expected value. Against the statistical expectation, liposomes could accumulate a remarkable number of solutes and forming a cell-like (crowded) compartments.

How was this possible? The mechanism is still unknown, but this phenomenon is very important for the origin of life scenario. Even starting from a diluted solution, the vesicles could entrap the solutes up to a very high concentration. In statistical terms, it was also surprising to discover that such phenomenon follows a “power low” distribution curve instead the expected Poisson distribution (de Souza et al., 2011), a sort of “all or nothing” scenario.

These studies are limited to those solutes that can be seen by electron microscopy, so it was not possible to see a kinetic reaction. With the present work, we aim at investigating more dynamic systems and using fluorescent molecules, so that biochemical reactions can be followed by confocal laser-scanning microscopy. We start from a single fluorescent protein to move then to a system formed by one enzyme and one fluorogenic substrate and see if the reaction can work better inside the liposomes than in diluted solution, to arrive then to a more complicated system in which 80 components are entrapped in a vesicle in order to express the green fluorescent protein (eGFP) using two different minimal kits.

2 Publication Overview

2.1 Physical Routes to Primitive Cells: An Experimental Model Based on the Spontaneous Entrapment of Enzymes Inside Micrometer-Sized Liposomes

Erica D'Aguanno , Emiliano Altamura , Fabio Mavelli , Alfred Fahr , Pasquale Stano and Pier Luigi Luisi

Life (Basel). 2015 Mar 18;5(1):969-96.

Abstract: How did primitive living cells originate? The formation of early cells, which were probably solute-filled vesicles capable of performing a rudimentary metabolism (and possibly self-reproduction), is still one of the big unsolved questions in origin of life. We have recently used lipid vesicles (liposomes) as primitive cell models, aiming at the study of the physical mechanisms for macromolecules encapsulation. We have reported that proteins and ribosomes can be encapsulated very efficiently, against statistical expectations, inside a small number of liposomes. Moreover, the transcription-translation mixture, which realistically mimics a sort of minimal metabolic network, can be functionally reconstituted in liposomes owing to a self-concentration mechanism. Here we firstly summarize the recent advancements in this research line, highlighting how these results open a new vista on the phenomena that could have been important for the formation of functional primitive cells. Then, we present new evidences on the non-random entrapment of macromolecules (proteins, dextrans) in phospholipid vesicle, and in particular we show how enzymatic reactions can be accelerated because of the enhancement of their concentration inside liposomes.

Own contribution on the article:

1) Performing Experiments
2) Data evaluation, interpretation and presentation of the results.
3) Contribution in writing the article

2.2 A remarkable self-organization process as the origin of primitive functional cells.

Pasquale Stano, Erica D'Aguanno , Jürgen Bolz , Alfred Fahr , Pier Luigi Luisi

Angew Chem Int Ed Engl. 2013 Dec 9;52(50):13397-400

Abstract: Encapsulation: The emergence of primitive cells remains an enigma of the origin of life. By modeling this key process as the encapsulation of a complex multimolecular mixture inside liposomes, a remarkable self-organization process has been revealed that brings about solute-rich compartments in which protein synthesis can take place.

Own contribution on the article:

1) Performing Experiments
2) Contribution in data evaluation, interpretation and presentation of the results.
3) Contribution in writing

2.3 Encapsulation of Ferritin, Ribosomes, and Ribo-Peptidic Complexes Inside Liposomes: Insights Into the Origin of Metabolism.

Tereza Pereira de Souza , Pasquale Stano, Frank Steiniger , Erica D'Aguanno , Emiliano Altamura , Alfred Fahr , Pier Luigi Luisi

Orig Life Evol Biosph. 2012 Oct 19;42(5):421-8.

Abstract: Here we summarize the main results of our latest investigation on the spontaneous encapsulation of proteins (ferritin) and ribosomes inside lipid vesicles. We show that when vesicles form in a solution containing some macromolecules (even at low concentration), in contrast to the expectations, a few but measurable number of vesicles is able to capture a very high number of solutes, up to 60 times the external concentration. We also show preliminary evidences on the encapsulation of additional solutes (ribo-peptidic complexes, fluorescent proteins and enzymes), and shortly present our current approach aimed at exploiting this phenomenon. In particular, we would like to reveal how the formation of compartments can trigger effective intra-vesicle reactions starting from diluted solutions. Although the mechanistic details for this phenomenon are still missing, we claim that these new evidences are highly relevant for the origin of the first functional cells in primitive times.

Own contribution on the article:

1) Preparation of Ribo-peptidic complexes.

2) Performing some experiments, namely the ones described in the section “work in progress”.

3 Publications

3.1 Publication 1

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Article

Physical Routes to Primitive Cells: An Experimental Model Based on the Spontaneous Entrapment of Enzymes inside Micrometer-Sized Liposomes

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Abstract: How did primitive living cells originate? The formation of early cells, which were probably solute-filled vesicles capable of performing a rudimentary metabolism (and possibly self-reproduction), is still one of the big unsolved questions in origin of life. We have recently used lipid vesicles (liposomes) as primitive cell models, aiming at the study of the physical mechanisms for macromolecules encapsulation. We have reported that proteins and ribosomes can be encapsulated very efficiently, against statistical expectations, inside a small number of liposomes. Moreover the transcription-translation mixture, which realistically mimics a sort of minimal metabolic network, can be functionally reconstituted in liposomes owing to a self-concentration mechanism. Here we firstly summarize the recent advancements in this research line, highlighting how these results open a new vista on the phenomena that could have been important for the formation of functional primitive cells. Then, we present new evidences on the non-random entrapment

of macromolecules (proteins, dextrans) in phospholipid vesicle, and in particular we show how enzymatic reactions can be accelerated because of the enhancement of their concentration inside liposomes.

Keywords: carbonic anhydrase; confocal microscopy; crowding; spontaneous concentration; enzymes; lipid vesicles (liposomes); primitive cells; proteinase K; self-organization

1. Introduction

It is accepted that life on Earth originated from the inanimate matter through a very long and slow series of steps which brought about the transformation from small molecular precursors to primitive cells, and from the latter to the last universal common ancestor (LUCA), from which biodiversity was then generated. The first part of this narrative is supported by a remarkable series of prebiotic chemistry experiments (reviewed in [1]), whereas the nature of the LUCA can benefit—up to a certain extent—of reconstruction of the roots of the evolutionary trees by molecular phylogenetics and bioinformatics. The central part of the story, namely, on how cells have been originated by separated molecules and on the origin of those functional molecules, is largely unknown and it is also interrupted by several conceptual gaps. Unanswered questions about the synthesis of sufficiently long macromolecules, about their specific sequences and functions, or about the origin of genetic code, or about the emergence of early self-replicating entities hinder the full understanding of cellular origin. Among these questions, one of the most important refers to the origin of primitive cellular structures that preceded both in time and complexity the early full-fledged biological cells.

We do not know what was the structure of primitive cells—which were probably not fully autonomous ("limping" cells [2,3]). Possibly they were lipid vesicle containing a sort of rudimentary metabolic and genetic systems.

If proteins and nucleic acids came first, then how were all macromolecules entrapped in a single compartment? If, on the other hand, functional macromolecules originated from inside the compartment that would mean that we then have to conceive semi-permeable, sophisticated membranes in prebiotic times, which does not appear plausible.

In recent years, stimulated by our original observations on protein encapsulation inside fatty acid vesicles [4,5] and by the reconstitution studies based on protein synthesis inside phospholipid vesicles [6–9], we started a direct investigation of solute entrapment during liposome formation, with specific attention to macromolecular solutes. Our results, which have been recently published [10–15], possibly offer a partial solution to the question about the origin of cellular structures, because they show that proteins and ribosomes can be encapsulated with high efficiency inside liposomes (actually, inside a small number of liposomes in a population).

In this article we would like to firstly review the main results of previous investigations, including a short historical development of the field, then move to new data on the encapsulation of enzymes and other macromolecules in lipid vesicles. These new experiments were designed and realized specifically to show the spontaneous formation of enzyme-rich vesicles—here considered as primitive cell models—with an internal "metabolic" activity, when compared with the same non-confined system

(the reaction in bulk). This provides a possible explanation to the origin of functional cells and at the same time opens a new vista on the principles of the entrapment of solute in vesicles (which might also be relevant for biotechnological applications).

2. The Minimal Cell and a Review of Previous Results on Macromolecules Entrapment in Liposomes (Part of the Discussion Derives from [13])

2.1. The Minimal Cell

Let us go back to the question, what kind of primitive cells emerged from prebiotic chemistry and that later brought to LUCA. The two possibilities—which are typically discussed—are protein-first and RNA-first scenarios. As commented in previous papers [3,13], both present advantages and disadvantages for a narrative of origin of life, mainly because these two classes of biopolymers excel in catalysis or in replication, and both are actually needed for constructing a living cell. The discovery of ribozymes [16,17] has greatly prompted a vision where RNA molecules alone were sufficient to achieve the needed cellular (and pre-cellular) functionality [18], and a RNA-protocell model has also been proposed [19,20], but several open questions on the origin of RNA world and on its evolution toward the LUCA's RNA/protein/DNA world, with no experimental and theoretical answers, still remain.

It is quite plausible to suppose, however, that a sort of simplified bounded molecular system existed before the origin of the first full-fledged cell, and that such system contained the minimal and sufficient number of molecular components to be defined alive, or at least to display some of the most relevant features of living cells, such as compartmentalized reactions, ribosomal protein biosynthesis, the capacity of self-maintenance, and possibly self-reproduction.

In the last years, we have been concerned with experimental studies on the "minimal cell" [3], focusing on the simplest and most ancient possible structure of biological cells. Our approach is shown in Figure 1: we incorporate extant genes and enzymes inside lipid vesicles—which function as model of cell membrane. In particular, the term "semi-synthetic minimal cells" has been used to describe minimal cells models that can be realized in the laboratory. This approach has multifold advantages. Firstly, minimal cells can be built in the laboratory since all components are available. Therefore, minimal cells belong to the realm of laboratory approaches. Secondly, this "synthetic" approach [3,21,22] allows testing hypothesis about the minimal complexity required for cellular life. In principle, in fact, it is possible to reconstruct and study the desired functions in a fully artificial system that mimics the cellular structures that existed billions of years ago. Thirdly, physical effects, not only (bio)chemical ones, can be investigated, such as the entrapment of solutes—this will be the main topic of this article. Finally, the methods and strategies applied for constructing minimal cells in origin of life scenario can be exported to other fields, such as synthetic biology, biotechnology, nanomedicine.

How did *primitive* minimal cells originate? Even if these structures have, by definition, a minimal genetic/metabolic complexity, it is evident that they must contain hundreds of components, just to count the macromolecular (function-bearing) ones. Here two possibilities can be discussed (Figure 2). The first one is that the biochemical network developed firstly in the environment, and later become encapsulated inside lipid vesicles; the second one is that the network was born already within compartments, starting from simpler molecules. Both appear difficult. The first one because it is

hundreds of macromolecules and small molecules should be encapsulated within the same lipid vesicle in order to have a functional cell; the second because during the (very long) process of network development, building blocks should enter the compartment, byproducts should leave it, and permeability should be somehow controlled in order to have such a sophisticated "bioreactor" that function correctly. Can experiments on *semi-synthetic* minimal cells—those that can be constructed in the laboratory—help to clarify, at least partially, such question?

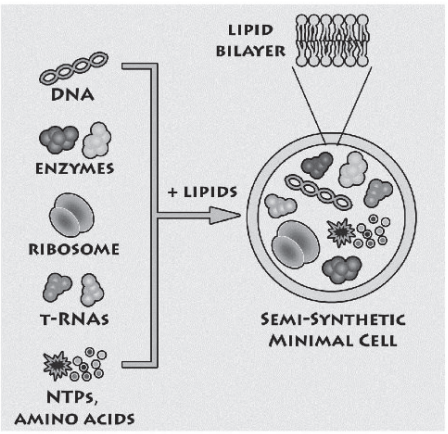

Figure 1. Experimental approach for constructing semi-synthetic minimal cell (reproduced from [23] with the permission of Elsevier).

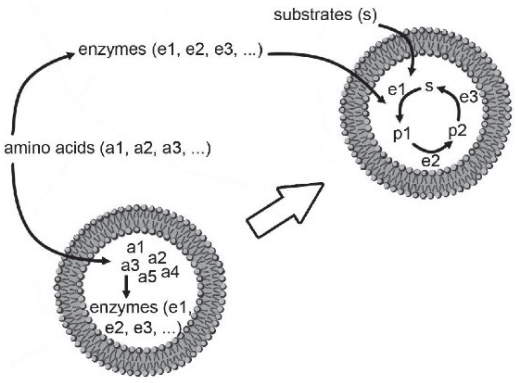

Figure 2. Two alternative (and perhaps competitive) hypothetical mechanisms for the formation of the first protocells, whereby the first proteins (and enzymes) were constructed inside a compartment (bottom), or first outside, then incorporated inside (top). Redrawn, with minor modifications, from [13].

2.2. Transcription-Translation Reactions inside Liposomes

Transcription-translation (TX-TL) network is the core set of reactions in minimal cells. The genes that encode such network constitute about 60% of the "minimal genome" [24,25], and TX-TL reactions can be carried out *in vitro* by using cell extracts (for example, from *Escherichia coli*) or

reconstituted systems. For these reasons, the construction of semi-synthetic minimal cell encapsulating TX-TL mixtures is a quite advantageous way to mimic primitive cells of minimal yet not negligible complexity. The PURE system (Figure 3A) is a reconstituted TX-TL kit composed by the minimal number of components (about 80 macromolecules, two dozen small molecules, organic buffer, and inorganic salts) required for synthesizing a protein starting from DNA [26,27].

The PURE system and cell extracts have been used to produce functional proteins inside lipid vesicles (results reviewed in [21]). Starting from the aqueous solution of the PURE system, lipid vesicles are formed *in situ*, for example by swelling phospholipid films or by adding lipids as ethanol solution. In these conditions, lipid vesicles form spontaneously and it happens that they mechanically entrap the macromolecular components of the TX-TL kit. In order to produce a protein, all PURE system components must be necessarily present in the same lipid vesicle. Experimental results obtained with conventional vesicles of diameter < 300 nm [9] show that this is indeed the case (Figure 3B), even if, at first sight, such multimolecular co-entrapment appears to be statistically implausible [2].

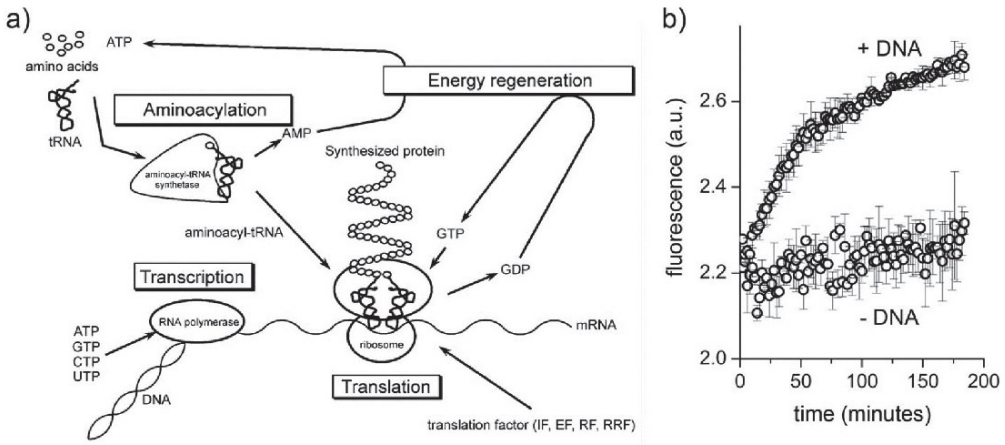

Figure 3. Protein synthesis inside conventional liposomes. (**a**) Components and functions of the PURE systems (reproduced from [27] with the permission of Elsevier); (**b**) fluorescence *versus* time profiles of eGFP producing vesicles (+DNA) and negative control (−DNA) (reproduced from [9] with the permission of Wiley).

Let us see the reasons. In order to make a quantitative estimate, it is possible to calculate the probability of simultaneous entrapment of all PURE system macromolecules inside lipid vesicles of a certain diameter according to standard theory. In particular, in agreement with the following null hypotheses (H_0): the entrapment can be modeled as a random sampling event, and the average number of solute molecules found in a vesicle of volume V is simply $\mu = N_A C_{bulk} V$, where N_A is the Avogadro's number and C_{bulk} is the bulk solute concentration. If this is true, it also follows that the probability of finding n molecules in a vesicle (when μ are expected) is given by the Poisson statistics, *i.e.*, $p(n) = e^{-\mu} \mu^n/n!$, and that the co-entrapment probability for k molecules is the product of the k individual entrapment probabilities [9]. As intuitively expected, the co-entrapment of one (or several copies) of each PURE system component is highly improbable inside small vesicles, and the probability

values are of the order of 10^{-26}. In other words, the results presented in [9] could be understood only by rejecting H_0. In particular, calculations have shown that experimental data could be explained by supposing a spontaneous concentration of PURE system components (*i.e.*, $C_{vesicles} > C_{bulk}$).

2.3. The Entrapment of Ferritin inside Liposomes and Other Recent Results

Intrigued by these unexpected conclusions, we then started a direct investigation on solute encapsulation inside spontaneously formed lipid vesicles. We recalled our previous work on the use of the protein ferritin as a marker for the vesicle lumen [4,5]. Individual ferritin molecules contain high amount of iron in form of hydrous ferric oxide phosphate, and it is widely used in electronmicroscopy. Since the number of ferritin molecules inside vesicles can be directly counted in images obtained by cryo-transmission electronmicroscopy (cryo-TEM), and the vesicle size is measurable as well, it is possible to measure the solute occupancy distribution $f(n)$ in vesicle populations and compare it with the expected Poisson distribution, $p(n)$. As shown in Figure 4A, the observed distribution is quite different than the theoretical one. The distribution is not bell-shaped, and—at high n—the measured distribution has a long "tail" of values that are significantly higher than the vanishing small values of the Poisson curve. The experimentally determined distribution strongly resemble a power law, *i.e.*, $f(n) \sim 1/n^a$ ($a > 0$), and Figure 4B summarizes in one micrograph the main message behind this study: as a result of spontaneous vesicle formation and solute encapsulation processes, empty and filled vesicles coexist in the same sample, and whereas it is evident that most of the vesicles contain a low number of ferritin molecules, or are empty, a minority of them (<1%) contains, against expectations, a very high number of ferritin molecules in non-aggregate state. The existence of these "super-filled" vesicles is considered almost impossible according to Poisson statistics, whereas is correctly predicted by the power law. Moreover, in the case of very small vesicles, exceptionally high intravesicle ferritin concentrations have been observed (up to about 300 μM), roughly corresponding to crowding concentrations in biological cells.

Experiments were repeated with ribosomes [11] and with peptidyl-RNA complexes [12], obtaining similar results. The scenario that is going to be disclosed is the following. When lipid vesicles, especially sub-micrometer ones, are formed in an aqueous phase containing macromolecular solutes, vesicle formation mechanisms and solute entrapment mechanisms bring about the formation of super-filled vesicles, as if solutes are sucked in, irrespective of the expected tendency of spreading in the largest possible volume. This happens, however, only for few special vesicles, probably those experiencing particular environmental local conditions that permit the onset of such a peculiar mechanism of solute encapsulation/vesicle formation. In absence of more detailed mechanistic information, we have made the hypothesis that such super-entrapment is based on the perturbation of the vesicle formation mechanism (*i.e.*, a kinetic effect; slowing down the closure of open lipid bilayers [28] due to solute-water-membrane interactions), whereas the driving force for the accumulation of molecules (*i.e.*, a thermodynamic effect) could be the cooperative release of bound water, as happens in the well-know hydrophobic effect.

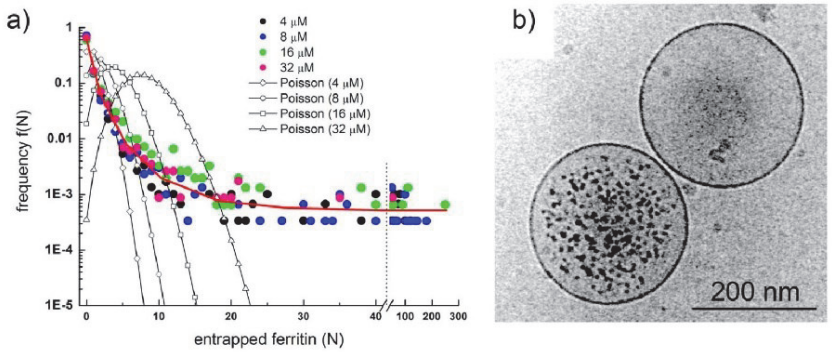

Figure 4. (**a**) Ferritin occupancy distribution as determined by analyzing about 7700 individual vesicles imaged by cryo-TEM. Experimental points shown as colored circles, theoretical expectations as empty circles connected by a line; (**b**) Two conventional POPC vesicles: one is super-filled with ferritin (encapsulating much more ferritin molecules than expected), the other does not contain any ferritin molecule (also against expectations). Images reproduced from [10] with the permission of Wiley.

Although more experimental and theoretical studies are required in order to verify this hypothetical mechanism (coarse-grained simulations seems to deny it [29,30]), its implications are clear: the formation of lipid vesicles has the potentiality of concentrating substances in their lumen. If this mechanism can be translated to origin of life scenario it means that when primitive cells were formed by self-assembly of membrane-forming compounds (very probably a mixture of amphiphiles, see [31]), the solutes present in the environment, and in particular macromolecular ones, could have been accumulated inside and this would have been the major factor for the origin of cellular metabolism. Very recently we have directly assayed this scenario by forming lipid vesicles in diluted (and therefore unreactive) PURE system. Green fluorescent protein synthesis was observed only inside (few) vesicles, suggesting that also in the case of multimolecular mixture a power law distribution ruled the solute encapsulation ([14]; Mavelli and Stano, manuscript in preparation). Together with cyro-electronmicroscopy observations of PURE system-filled vesicles [11], this could explain our original observation of protein synthesis inside small conventional liposomes [9] in terms of simultaneous concentration of several macromolecules.

3. New Results on the Encapsulation of Proteins and Dextrans in Vesicles

Having summarized the most relevant results on the investigations of macromolecular entrapment inside lipid vesicles, let us illustrate the motivations behind the approach presented here, and show new results.

Key evidences about the accumulation of macromolecular solutes inside liposomes have been achieved via cryo-TEM by using large unilamellar vesicles (typical diameter: 100–400 nm) and solutes that can be visualized by this technique (ferritin, ribosomes, peptidyl-RNA complexes). With this approach it is possible to visualize vesicles individually, to directly see their shape and lamellarity, and count individual molecules. However, there are also two limitations, namely the impossibility of following in

real time biochemical reactions, and the quite narrow choice of solutes that can be visualized. For these reasons, a more versatile methodology would be helpful to extend the solute entrapment studies. Three possibilities are: confocal fluorescence microscopy, infrared microscopy [32], and flow cytometry [33,34].

We decided to extend our investigations by using confocal fluorescence microscopy. Fluorescence microscopy is particularly suitable for large vesicles (diameter > 0.4–0.5 μm), and can be used to assess the presence of solutes whose concentration can be measured, in direct or indirect way by a fluorescence signal. Fluorescently-labeled macromolecules or fluorescence-based enzymatic reactions are suitable for these purposes. We aim at studying the encapsulation of macromolecules like proteins, polysaccharides, and nucleic acids (D'Aguanno, manuscript in preparation). Both non-reacting and reacting systems will be presented here. These experiments will ultimately show that the formation of lipid vesicles might act as a sort of "attractors" for molecules present at low concentration in the environment. Such molecules, once encapsulated (and concentrated) inside vesicles, might overcome a concentration threshold that might trigger chemical reactions.

3.1. Liposome Preparation

In order to study the entrapment (encapsulation) of solutes in spontaneously formed vesicles we have chosen vesicle preparation methods that model as better as possible self-organization pathways, with minimal guidance by the operator. As lipids, we have employed the well-known 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), alone or in the presence of sodium oleate (20–50 mol%). Note that a similar study based on pure fatty acid vesicles—which are more realistic models of primitive cells—is currently in progress in our laboratory, and it will be published in a dedicated article (D'Aguanno, manuscript in preparation). We are aware of the fact that pure POPC vesicles probably do not represent the best primitive compartments model—although it has been, and is, used frequently, either because POPC is a phospholipid (whereas primitive lipids were simpler molecules, like fatty acids or isoprenoids), either because a *mixture* of simpler lipids, heterogeneous in terms of chain length and head group, are more plausible as components of early membranes. We will come back to this point in the concluding remarks.

We have optimized the preparation methods in order to obtain vesicles in the micrometer range (95% of vesicle diameters lies in the 0.83–2.5 μm range), so that their visualization by a common confocal microscope is non-ambiguous. The present study therefore differs from previous ones based on cryo-TEM (vesicle diameter < 0.4 μm) because it assays the encapsulation within larger vesicles. It also differs from a published study based on confocal microscopy [35] because the latter was focused on encapsulation in larger (giant) vesicles (vesicle diameter: 10–20 μm).

Method 1 (M1): *hydration of lipid film* [36]. This traditional vesicle preparation method simply consists in hydrating, with an aqueous solution, a previously dried lipid film. Generally the film is obtained starting from a solution of lipids in chloroform or similar solvent. In this study, for the matter of convenience (hydration with small volumes), we have deposited the lipid film over 2 mm glass beads, similarly to a previous report [37].

Method 2 (M2): *hydration of freeze-dried lipid vesicles* [38,39]. This method is similar to the simple film hydration, but instead of using a lipid film, 400 nm (or 800 nm) extruded and then freeze-dried lipid vesicles are used.

Method 3 (M3): *ethanol injection method* [40]. Lipid vesicles can be prepared by injecting a concentrated lipid solution in ethanol (or other water-soluble alcohols) into an aqueous solution. The size and morphology of resulting vesicles depend mainly from the type of lipids and from the concentration of lipids in the stock alcoholic solution [41,42]. In particular, large vesicles can be obtained when stock solutions of high concentration (100 mM) are used.

By all methods, we obtained vesicles in the micrometer range. In the case of M1, for example, vesicles with an average diameter of 1.3 μm (standard deviation ±0.6 μm, n = 427) and a wide size distribution (up to 5–6 μm) have been observed (Figure 5). In the case of M2 and M3, we obtained, respectively, fluorescent vesicles with diameters of 1.2 ± 0.3 μm (n = 48) and 1.4 ± 0.5 μm (n = 55).

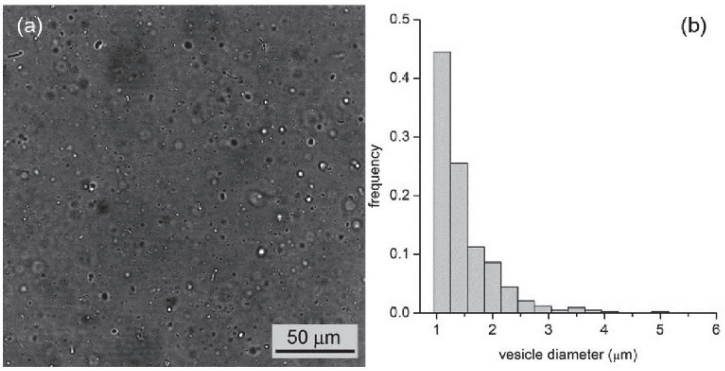

Figure 5. (**a**) Bright-field microscopy image of a vesicle sample as obtained by the film hydration method (M1); (**b**) Vesicle size distribution with an average diameter of 1.3 μm.

3.2. Entrapment of Proteins and Dextrans

Next, we have investigated the entrapment of solutes inside micrometer-sized vesicles prepared by the abovementioned three methods. We have followed a simple and straightforward procedure, namely preparing a population of lipid vesicles in an aqueous solution that contain a solute of interest at a certain concentration (typically between 0.1 and 5 μM). Then we simply analyzed the so-obtained samples by confocal microscopy (all tested solutes were fluorescent). If the solute molecules, once entrapped, have the same concentration as the bulk solution, liposomes will not be distinguished from the background. If, on the other hand, more solute molecules are entrapped inside liposomes, these will appear more fluorescent than the background.

Preliminary calibration experiment served (*i*) to build a calibration line for converting fluorescence to concentration (this was done for different instrumental settings, including gain and offset); and (*ii*) to ensure that the fluorescence values in the accessible range scaled linearly with fluorochrome concentrations, meaning that the assayed concentration range did not suffer of self-quenching and/or inner filter effects.

We have employed common commercially available fluorescent proteins and dextrans with different molecular weights, in particular: dextran conjugated with rhodamine (dextran-RITC, *ca.* 10 kDa), phycoehrytrhin (PE, *ca.* 240 kDa), bovine serum albumine conjugated with fluorescein (BSA-FITC,

ca. 66 kDa), allophycocyanin (APC, *ca.* 104 kDa), and dextran conjugated with fluorescein (dextran-FITC, *ca.* 150 kDa).

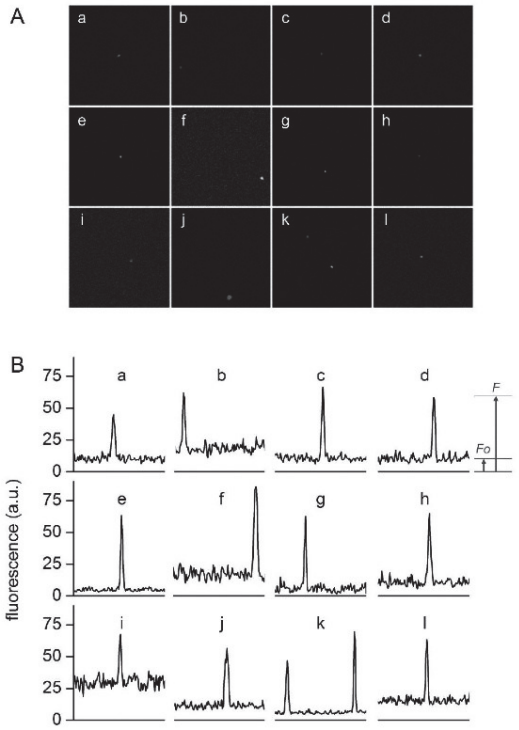

Figure 6. Typical confocal images (**A**) and fluorescence profiles; (**B**) of lipid vesicles whose fluorescence exceeds the fluorescence of the external solution. Solutes: Dextran-FITC (a, b, i), BSA-FITC (c, d, f, g, j, k), dextran-RITC (e), APC (l), PE (h). Methods: M1 (b, e, g, h, i-l), M2 (c, d), M3 (a). The diameters (μm) and the r_F values of the vesicles are, respectively: (a) 1.3, *4.3*; (b) 1.2, *3.4*; (c) 1.8, *6.1*, (d) 1.5, *6.4*; (e) 1.4, *13*; (f) 1.3, *6.1*; (g) 1.2, *10.6*; (h) 1.3, *6.3*; (i) 2.7, *2.3*; (j) 1.0, *5.5*; (k) 1.5, *7.3* and 1.2, *10.8*; (l) 1.4, *4.2*.

Figure 6 shows a set of typical images obtained by confocal microscopy. Similar images have been obtained for all macromolecular solutes investigated in this work. The results can be summarized as follows. When solutes alone were imaged, a homogeneous fluorescence was always observed. Aggregates were observed only in very rare cases. When vesicles were prepared in the presence of solutes, in all methods and for all lipid concentrations, and for all solutes, we have always observed a small but significant number of vesicles (somehow visible also in bright field) whose fluorescence was higher than the background. If we call F_0 the background fluorescence, and F the vesicle fluorescence, it is possible to define, for each visible vesicle, an in/out fluorescence ratio $r_F = F/F_0$ that indicates how many times intra-vesicle fluorescence is higher than background fluorescence, and therefore how many times solutes have been concentrated inside a certain vesicle. As evidenced by carrying out control experiments with fluorescent-labeled lipids (Figure 7), the great majority of vesicles are not visible

because their internal fluorescence is very near F_0 (indicating that they have encapsulated the expected number of solutes). On average, less than 1%–2% of vesicles were more fluorescent than the background.

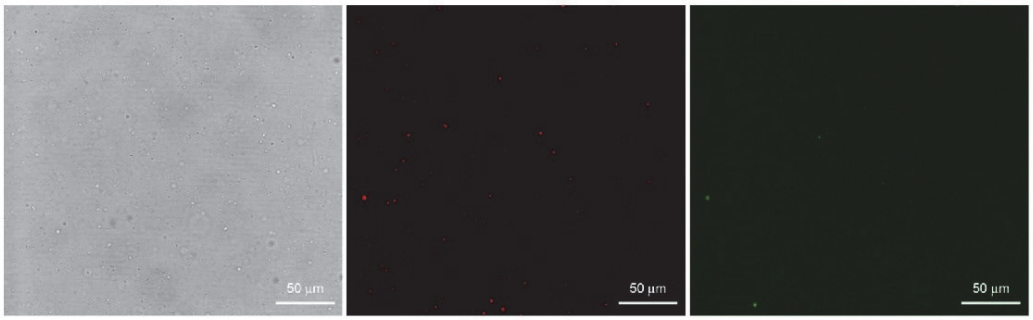

Figure 7. Bright field (**left**) and fluorescence confocal images (**center**, **right**) of lipid vesicles prepared by the film hydration method (M1) starting from a FITC-dextran solution. Vesicles were made of POPC and 0.05 mol% DOPE-lissamine-rhodamine in order to be red-fluorescent (central image). Note that despite the abundant presence of vesicles, only few of them have encapsulated a high amount of green-fluorescent solute (right image) so to appear more fluorescent than the background.

The diameter d (µm) of the fluorescent vesicles can be estimated by simply considering that the circular (or quasi-circular) area imaged by confocal scanning is a proxy for the vesicle great circle. Being the optical thickness *ca.* 0.9 µm in our experimental setup, and comparing the vesicle size as appears in bright field images, this approximation appears a viable one.

We have summarized the entrapment results in Table 1. Actually, each experiment was run several times by changing details of the experimental method (for example: lipid concentration, solute concentration, buffer). However, data have been pooled together because despite the variations of the experimental conditions, the differences between the r_F values of each dataset were not statistically significant ($p > 0.05$).

A detailed analysis of the experimental data clearly reveals that there is no correlation between the factors r_F and the vesicle size, whereas a conclusion on the possible correlation between r_F and bulk solute concentration remains elusive in this study (statistical analysis provides inconsistent results).

On average, the $\langle r_F \rangle$ values lie around 3–4, with some exceptions (5.7, 6.1, 12). This was not unexpected because of the very stochastic nature characterizing the process under study. This behavior (occasional occurrence of high in/out ratios) has also been found in kinetic experiments (see below) as well as in previous studies [9–11].

In all cases, the fluorescence distribution is asymmetric (with positive skewness values up to about 2.8), as summarized in Figure 8. This means that there are, on the right-hand side of these distributions, r_F values that are higher than the average. These maxima ($r_{F,max}$) are also reported in Table 1, along with the mean r_F values. These values refer to rare vesicles whose fluorescence is rather high when compared not only to the fluorescence background, but also to the other vesicles. In other words, these vesicles lie at the extreme of the vesicle fluorescence distribution. Note that these very bright vesicles (with $r_F > \langle r_F \rangle$) represent a fraction of 0.01%–0.02% of the entire vesicle population.

Table 1. Average and maximal r_F values for different solutes and vesicle preparation methods.

Solute	MW (kDa)	Lipids	Method [c]	$\langle r_F \rangle \pm$ SD (n) [d]	$r_{F,max}$	Note
Calcein	0.67	POPC	M1	n.a.	n.a.	– [e]
Dextran-RITC	10	POPC	M1	12 ± 3.8 (11)	19.8	– [f]
BSA-FITC	66	POPC	M1	5.7 ± 4.5 (69)	29	– [f]
BSA-FITC	66	PB33PEO29 [a]	M1	2.9 ± 0.2 (19)	3.2	– [g]
BSA-FITC	66	POPC	M2	3.7 ± 1.4 (48)	7.7	– [g]
BSA-FITC	66	POPC ± oleate [b]	M3	3.5 ± 1.1 (13)	8.6	– [f]
APC	104	POPC	M1	3.9 ± 1.6 (19)	7.3	– [h]
Dextran-FITC	150	POPC	M1	2.7 ± 1.0 (15)	5.1	– [f]
Dextran-FITC	150	POPC ± oleate [b]	M3	3.8 ± 1.1 (55)	6.4	– [f]
PE	240	POPC	M1	6.1 ± 2.3 (11)	9.9	– [i]

[a] Copolymer vesicle made by Poly(butadiene-b-ethylene oxide), average MW 1.85 kDa; [b] POPC:oleate has been varied as 100:0, 80:20, 50:50 (without observing any statistical difference among the samples); [c] M1—hydration of thin lipid films deposited on glass beads; M2—hydration of freeze-dried lipid vesicles; M3—ethanol injection method. [d] $\langle r_F \rangle$, SD, and n stand for, respectively, the mean value, the standard deviation, and the number of observations (number of vesicles). [e] No vesicles with fluorescence higher than the background were systematically observed; [f] $[\text{solute}]_{bulk}$ from 0.63 to 5 μM, intravesicle concentration not dependent from vesicle size and solute concentration; [g] $[\text{BSA-FITC}]_{bulk}$ = 4 μM, intravesicle concentration not dependent from vesicle size; [h] $[\text{APC}]_{bulk}$ from 0.5 to 5 μM, intravesicle concentration not dependent from vesicle size, yet dependent from solute concentration in inverse way; [i] $[\text{PE}]_{bulk}$ from 0.16 to 0.25 μM, intravesicle concentration not dependent from vesicle size and solute concentration; "n.a." stands for "not available".

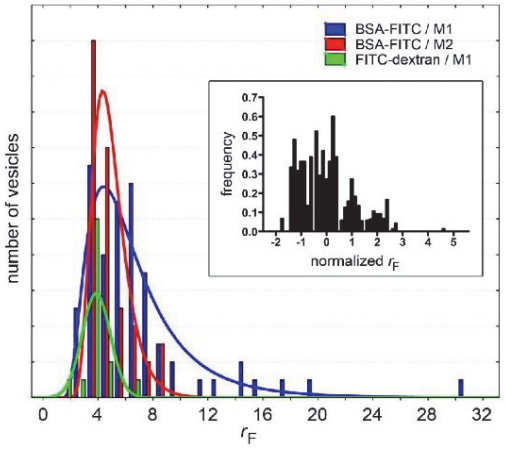

Figure 8. Fluorescence distributions of solute-containing vesicles (only cases with $n > 48$ have been shown, see Table 1). Note that whereas for BSA-FITC the distributions can be fitted with a log-normal curve (red and blue sets), FITC-dextran-containing vesicles have an approximately normal r_F distribution (green). Inset: To compare different populations (all solutes in Table 1), the distributions of normalized r_F values are shown; where $r_{F,normalized} = (r_F - \langle r_F \rangle)/SD$. It should be noted, however, that since these distributions have been built with a small number of events, their interpretation should be done with caution.

Importantly, in analogous experiments with the low molecular weight solute calcein (0.67 kDa, used at a concentration of 1.25–10 μM) it was not possible to detect vesicles with fluorescence higher than the background.

3.3. Entrapment of Enzymes—Rate Enhancement

Having shown that the concentration of macromolecules spontaneously entrapped inside some liposomes can be higher than the external concentration by a factor r_F, the next step is to show how this phenomenon impacts on chemical reactions inside liposomes.

Clearly, in those liposomes where the concentrations of chemicals are higher than in the environment, a faster reaction rate is expected. This scenario can be experimentally tested with simple enzyme reactions (we have already seen that in the case of complex, multi-step reactions, such as the protein synthesis, the spontaneous concentration of the macromolecular TX-TL machinery can even trigger the otherwise undetectable or not-occurring protein synthesis).

Here we have developed two simple scenarios: (*i*) an enzyme is encapsulated within vesicles, and a permeable, low-MW substrate is added afterward; (*ii*) an enzyme and a macromolecular substrate are co-encapsulated within vesicles, and reaction occurs immediately.

The data shown in Section 3.2 suggest that the super-concentration effect described in this paper is evident especially for macromolecules. Enzymes are macromolecules and therefore it is expected that when a population of vesicles is formed in an enzyme solution, some of them (usually <1%) will contain a number of enzyme molecules higher than expected. For vesicles of 1–2 μm in diameter, concentration factors around 2.7–6.1 are typically obtained for a variety of solutes (proteins, dextrans). Such values can be used as an educated guess for estimating the behavior of a generic protein that does not establish strong and specific interaction with lipids.

Figure 9 shows two Michaelis-Menten plots referring to a hypothetical case of an enzyme that is concentrated three times when encapsulated within vesicles ($[E]_2/[E]_1 = 3$). At a fixed substrate concentration $[S]_1$ (in this example, $[S]_1 = K_M/2$), an initial rate enhancement of a factor 3 is expected (Figure 9, blue line from P to Q). Note that such a factor is actually independent from the choice of $[S]_1$). This example represents the case of macromolecular enzyme and low MW solute, discarding for the moment the problems of substrate permeability rate across the membrane (for a treatment, see below).

If the substrate is also a macromolecule, its encapsulation occurs simultaneously with the enzyme. It can happen that both macromolecules (the enzyme and the substrate) are simultaneously concentrated each by a factor three inside some liposomes ($[E]_2/[E]_1 = 3$, $[S]_2/[S]_1 = 3$). As shown in Figure 9 (red line from P to R), the initial rate enhancement would be of a factor 5.4. Note that in this example $[S]_1 = K_M/2$ and $[S]_2 = 3K_M/2$, *i.e.*, the substrate concentration moves from below to above K_M. The effect here is not so dramatic because we have supposed a low concentration factor (3×), but if a similar mechanism would increase the substrate concentration by a factor 10 or so—as it happens in conventional sub-micrometer vesicles [9]—one could observe a quite significant rate enhancement inside (few) vesicles.

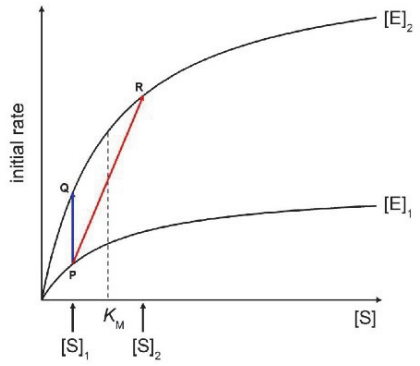

Figure 9. Effects of increasing enzyme and substrate concentration on initial rates of Michaelis-Menten kinetics. See text for details.

Here we have studied two experimental systems that recapitulate the two cases illustrated above. In particular, case (*i*) has been modeled with the reaction between carbonic anhydrase and carboxyfluorescein diacetate, whereas for case (*ii*) proteinase K and BSA-FITC have been employed.

3.3.1. Carbonic Anhydrase (CA) and Carboxyfluorescein Diacetate (CFDA)

It is well known that carbonic anhydrase (CA) catalyzes other reactions besides the reversible hydration of CO_2, in particular, the hydrolysis of esters has been reported [43], as the case of *p*-nitrophenyl acetate [44]. We have employed the membrane-permeable substrate 6-carboxyfluorescein diacetate (CFDA) to carry out an enzymatic reaction inside CA-containing liposomes. Notably, the product of the reaction (6-carboxyfluorescein, CF) is membrane impermeable.

The experimental plan is as it follows. Liposomes are formed in a solution containing CA, so that some of them will presumably contain an excess number of CA molecules due to spontaneous super-concentration of this macromolecule (29 kDa) in the liposome lumen. Next, CFDA is added and it will react both with free (external) and encapsulated CA, with the caveats that in the latter case CFDA shall first permeate the liposome membrane (Figure 10). Consequently, a comparison between the rates of bulk and liposomal reaction will reveal whether and what extent super-filled CA-containing liposomes form spontaneously.

Experimental results are shown in Figure 11. It is possible to follow the time course of CFDA hydrolysis by confocal microscopy, recording the fluorescence increase at different times, both in bulk than inside liposomes. Figure 11a shows typical microscopic images referring to 0, 10, 20 and 30 min after CFDA addition to CA-containing liposomes (note that free, non-entrapped CA was not removed, so that the fluorescence background also increases in time). By measuring the fluorescence of the liposomes and of the background (Figure 11b,c), at each sampled time, it is possible to plot the rate of enzyme reaction in bulk and inside liposomes. Note that because the liposomes freely float in the solution, it was not possible to follow their individual behavior (namely, the fluorescence increase inside a certain liposome). We therefore captured images containing a number of liposomes and average their fluorescence (the average value, however, is affected by a bias because it is difficult to spot out liposomes that are only slightly more fluorescent than the background; this means that most of

the pictures generally contain the brightest liposomes of the population). The fluorescence-*versus*-time profiles (inside liposomes and in bulk) are shown in Figure 12a.

All experiments gave qualitatively similar results, although the numerical reproducibility is hindered by the stochastic nature of the events we are focusing on. Summarizing:

- free (non-encapsulated) CA catalyses the hydrolysis of CFDA, and therefore the background fluorescence increases with a specific rate (V_{bulk}, in fluorescence units/second) corresponding to the bulk reaction; and
- few liposomes randomly appeared in the illuminated field as bright spots indicating that inside those liposomes CFDA permeated inside the aqueous lumen and reacted with encapsulated *CA*. Being brighter than the background, the amount of CF produced per unit of volume (inside liposomes) is higher than the corresponding quantity in bulk. This intraliposome reaction rate is indicated as $V_{liposome}$ (fluorescence units/second).

The ratio r between these rates ($r_V = V_{liposomes}/V_{bulk}$) mirrors, at first approximation, the ratio between the CA concentration inside liposomes and in bulk ($[CA]_{liposome}/[CA]_{bulk}$), (but see below for a more detailed treatment). It is then possible to estimate the overconcentration of CA inside liposomes in the moment of their formation by measuring the ratio r_V.

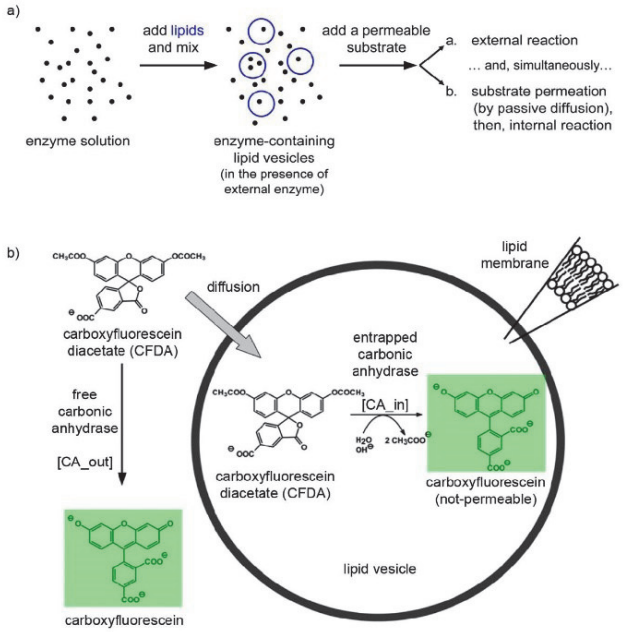

Figure 10. (**a**) Experimental strategy for co-encapsulating an enzyme (carbonic anhydrase, CA, black spots) inside lipid vesicles, followed by adding a membrane-permeable substrate (6-carboxyfluorescein diacetate, CFDA). Note that the external enzyme molecules have not been removed and can react outside vesicles. (**b**) Details of the system under study, showing that CFDA can either react outside vesicles with free CA either permeate through the lipid membrane, reach the vesicle lumen, and then react with the encapsulated CA. The green-fluorescent product, 6-carboxyfluorescein, has been marked by a green box.

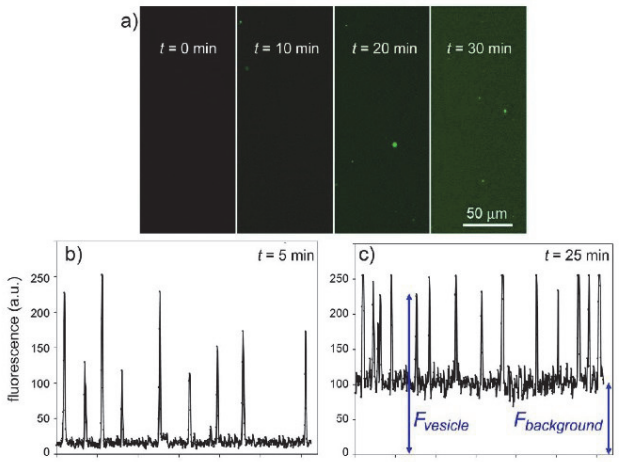

Figure 11. Experimental results for the CA + CFDA system. (**a**) Confocal micrographs showing green fluorescent vesicles that appear in the progress of reaction (t = 0, 10, 20, 30 min). The vesicles appear more fluorescent than the background although the same reaction is also occurring outside vesicles. (**b**,**c**) The vesicle fluorescence can be estimated by image analysis (by ImageJ software [45])—here shown as the pixel luminosity of samples taken at 5 and 25 min after mixing. Note that the fluorescence values of each vesicle greatly differ from each other (giving a fluorescence distribution, *cf.* Figure 8) and are well above the background. Note also that the background fluorescence increases with time, as expected.

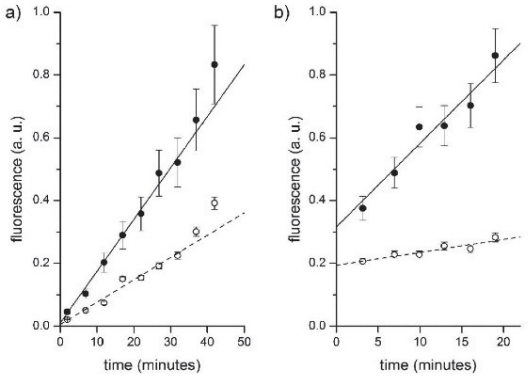

Figure 12. Fluorescence *versus* time profiles of enzymatic reactions inside vesicles (filled circles) and in bulk (empty circles). (**a**) CA-containing vesicles plus externally added CFDA. Data have been fitted by straight lines; the ratio between slopes being 2.3. (**b**) Proteinase K and BSA-FITC containing vesicles. Data have been fitted by straight lines; the ratio between slopes being 6.5.

As reported in a previous study [14], it is possible to estimate the fraction of liposomes that appear brighter than the background by doping the liposome membrane with fluorescent lipids (this is

necessary because the micrometer-sized liposomes used in this study are not well visible in bright field, phase contrast, or Nomarski acquisition modes).

Table 2 summarizes the experimental outcomes from a set of runs carried out by using different CA and CFDA concentration as well as different liposome preparation methods. Table 2 reveals a certain variability among the r_V values of different samples, but the pattern is similar to that recorded in the case of single fluorescent solutes (Table 1). In most cases, the ratio r_V between internal and external reaction rate, which is a measure of the ratio between the internal and external CA concentrations, lies in the 2–4 range. In particular cases, higher values are obtained (e.g., 8.7, 9.1, 11.4). This is due to the very stochastic nature of the phenomenon under study. The difficulty of controlling all microscopic variables affecting the encapsulation process (as well as the sampling procedure) results in a great variability among the experimental outcomes, which had, strictly speaking, a poor reproducibility (in rigorous quantitative sense). Qualitatively, however, all experiments clearly show a common pattern.

Table 2. Reaction between encapsulated CA and externally added CFDA.

[CA], μM	[CFDA], μM	Lipids	Method	r_V	Note
0.5	40	POPC	M1	11.4	– [a]
1.0	40	POPC	M2	2.0	– [b]
1.0	80	POPC	M2	3.6	– [c]
0.5	40	POPC/POPG 4/1	M1	2.1	– [d]
0.5	80	POPC/POPG 4/1	M1	2.3	– [d]
0.5	160	POPC/POPG 4/1	M1	1.9	– [d]
0.25	80	POPC/POPG 4/1	M1	8.7	– [d]
1.0	80	POPC/POPG 4/1	M1	1.3	– [d]
1.0	40	POPC/oleate 95/5	M3	2.9	– [e]
0.25	40	POPC/oleate 4/1	M3	9.1	– [f]
0.5	40	POPC/oleate 4/1	M1	2.5	– [d]

[a] [POPC] = 0.5 mM; [b] [POPC] = 5 mM, pre-extruded with membranes having pores of 800 nm in diameter; [c] [POPC] = 2.5 mM, pre-extruded with membranes having pores of 800 nm in diameter; [d] 200 mM HEPES (pH 7) was used as buffer, in combination with lipid film hydration without glass beads, and [POPC] = 0.5 mM; [e] 5 mol% oleate helps not to forming too small vesicles; [f] 400 mM sodium phosphate (pH 7) was used as buffer.

3.3.2. Numerical Simulations of the CA/CFDA System

In preliminary experiments, we have determined the apparent K_M and k_{cat} values for the reaction, being, respectively, 4.0 ± 0.3 mM and 3.0 ± 0.2 s^{-1}. The permeability coefficient of CFDA across the phosphatidylcholine liposomal membrane, namely 10^{-7} cm/s, has been taken from the literature [46]. It is therefore possible to simulate numerically the outcome of experiments as described in Section 3.3.1, namely, the addition of CFDA to CA-containing vesicles, in the presence of non-encapsulated CA.

The system was modeled by dividing the macroscopic sample volume in small "unitary" volumes of *ca.* 50 fL, each containing one liposome (d = 1.3 μm; liposome volume = 1.15 fL). These values have been estimated on the basis of the real experimental system. The starting point is a population of CA-containing liposomes suspended in a medium that still contains non-entrapped CA (0.5 μM). Since the enzyme CA is present both inside and outside the liposomes, it is convenient to analyze the system

by using the ratio $r_{CA} = [CA]_{in}/[CA]_{out}$, which can be easily varied, in order to explore three regimes: (*i*) $r_{CA} < 1$ when CA encapsulation is poor; (*ii*) $r_{CA} = 1$ when CA is equally present inside and outside vesicles; (*iii*) and $r_{CA} > 1$ when CA is over-concentrated inside liposomes. CFDA (80 μM), present externally, can react with the external CA according to Michaelis-Menten kinetics, so that the fluorescent carboxyfluorescein (CF) is produced in bulk. In a competitive process, CFDA penetrate inside liposomes through the membrane, and then react with internalized CA. We assume that CF cannot escape from the liposome (the permeability of carboxyfluorescein is negligible for the purposes of this model [47]).

Figure 13 shows the calculated kinetic profiles (30 min) for the production of CF in liposomes having $r_{CA} = 0.33$, 1, or 3. When $r_{CA} = 0.33$, the increment of CF concentration, and therefore of its fluorescence is faster in bulk than inside liposomes. The calculated ratio r_V between the slopes (V_{in}/V_{out}) in the quasi-linear region is ~0.28, mirroring in good way the model's r_{CA} value (0.33). When $r_{CA} = 1$ the CF concentration profiles differ only for the presence of a lag phase, due to the retardation effect exerted by the membrane. After the lag phase, in the quasi-linear region, the calculated ratio $r_V \sim 0.82$. Finally, when $r_{CA} = 3$ the increase of CF concentration is faster inside liposomes (after a lag phase). According to the model, CA-rich liposomes should soon appear more fluorescent than the background because of a faster accumulation of CF, despite the retardation effect due to the fact that CFDA must cross the membrane before reacting with encapsulated CA. In this case, the calculated r_V value is ~2.1, which is a proxy value for the true r_{CA} value ($r_{CA} = 3$) [48].

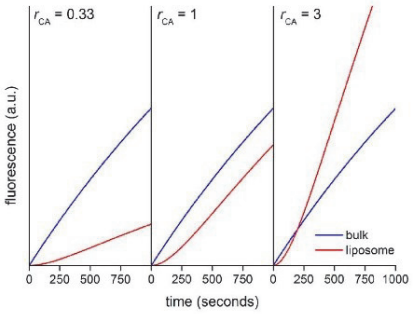

Figure 13. Calculated kinetic profiles for the enzymatic production of carboxyfluorescein from CFDA, under CA catalysis, inside and outside *in silico* vesicle with diameter of 1.3 μm.

3.3.3. Proteinase K and Bovine Serum Albumine-FITC (BSA-FITC)

Fluorescein isothiocyanate-labeled proteins have been used as substrates for proteases [49]; for instance BSA-FITC can be used for this purpose [50]. The commercial BSA-FITC used in this study carries an average of 9.5 FITC moieties, and has relatively low background fluorescence due to autoquenching. Proteolytic digestion alleviates auto-quenching and therefore brings about a concomitant fluorescence increase. This phenomenon provides the basis for a proteolytic assay (with proteinase K) whereby the increase in fluorescence is proportional to the degree of BSA-FITC degradation.

Firstly, we characterized and validated the reaction between proteinase K and BSA-FITC. By comparing the emission spectra of BSA-FITC before and after proteinase K treatment (18 h, 25 °C,

pH 7), a fluorescence increase of about 13 times is observed, roughly corresponding to the dequenching of about 3 out of the 7–12 FITC moieties bound to BSA-FITC. It is therefore possible to follow the course of the reaction quite accurately by confocal fluorescence microscopy. On the basis of kinetic analysis, and by using an apparent K_M ~ 2.3 mM (calculated by us from [50], and further confirmed by other studies based on synthetic peptides as substrates, see [51,52]), we then estimated an apparent k_{cat} value of about 1 s^{-1}.

Next, we carried out the co-encapsulation experiment, consisting in preparing a mixture of proteinase K (1 μM) and BSA-FITC (2.5–5.0 μM), and forming liposomes *in situ* (Figure 14). The idea is that because both reactants are macromolecules, both can spontaneously concentrate inside liposomes during the membrane closure. For practical reasons (limit of detection), the concentration of BSA-FITC (the substrate) cannot be reduced too much, so that the actual experimental setup does not strictly correspond to the example illustrated in Figure 9 (self-concentration of the substrate from a value below to one above K_M), but it just follows the same principles.

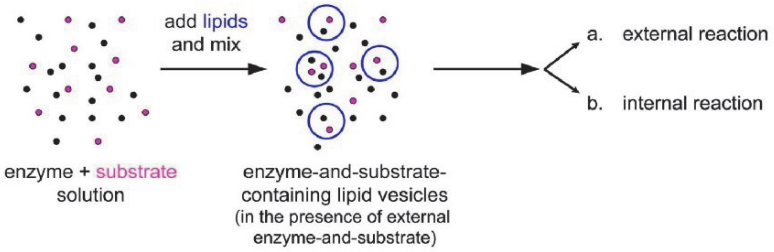

Figure 14. Experimental strategy for co-encapsulating an enzyme (proteinase K, black spots) and a macromolecular substrate (BSA-FITC, pink spots) inside lipid vesicles. Note that the external solutes have not been removed and can react outside vesicles.

Figure 12b and Table 3 summarize the typical results of the BSA-FITC/proteinase K system. Note that only few vesicles could be detected. Their mean fluorescence *versus* time profile has been reported and fitted with a straight line. The background fluorescence increase has also been recorded. The ratio between these two trends is around 6.5 (first entry of Table 3). Note that the intercepts of these two lines, in this and in other experiments, not always converge to the same value. This could be due to the fact that the sampled vesicles actually contain a higher internal BSA-FITC concentration, which further accelerates the internal reaction. Accordingly, the ratio between the intercepts (in Figure 12b, such ratio is 1.6) is a measure of the ratio between the internal and external BSA-FITC concentration in those vesicles that have been sampled.

Table 3. Reaction between co-encapsulated proteinase K and BSA-FITC.

[proteinase K], μM	[BSA-FITC], μM	Lipids	Method	r_V	Note
1	2.5	POPC	M1	6.5	– [a]
1	5	POPC	M1	2.2	– [a]
1	2.5	POPC	M2	11.6	– [b]
1	5	POPC	M2	7.3	– [b]

[a] [POPC] = 0.4 mM; [b] freeze-dried vesicles obtained from 800 nm-extruded POPC vesicles, [POPC] = 5 mM.

3.3.4. Statistical Considerations

Our data show that macromolecular solutes can be spontaneously encapsulated inside lipid vesicles at a concentration higher than the expected one. The ratio between the actual and the expected concentration (as measured by the r_F and r_V values, see Tables 1–3) typically lies in the 2–4 range, although r values higher than 10 have been also occasionally recorded.

The question is whether such "small" concentration factors (from 2 to 4) can be explained by invoking the natural stochastic fluctuations of the number of solute molecules that can be encapsulated within a vesicle. At this aim we recall our null hypothesis H_0 (see Section 2.2) saying that the fluctuations around the average number N_0 of solute molecules that are encapsulated within a vesicle of volume V are ruled by a Poisson distribution. Can stochastic fluctuations account for having an actual number N of encapsulated molecules so that $N > N_0$?

Firstly, we calculate N_0 as usual ($N_0 = N_A\, C_0\, V$), focusing on the experimental conditions giving the lower N_0 value—and stochastic factors are amplified. If natural fluctuations cannot explain data in these conditions, then even more so they do not in all other cases. When 1 μm (diameter) vesicle forms in a C_0 = 0.1 μM solution (the smallest concentration used in this study), $N_0 \sim 32$. This value (32) means that the Poisson distribution can be approximated by a Gaussian distribution. Fluctuations theory predicts that the magnitude of the fluctuations (ΔN) goes as $\sqrt{N_0}$, *i.e.*, $\Delta N \sim \sqrt{32} = 5.6$. We can then consider a Gaussian distribution of the number of entrapped solute molecules N, having average N_0 = 32 and standard deviation ΔN = 5.6, and ask what is the probability p to find vesicles with $N > r\, N_0$, for $r \geq 1$. Results are shown in Table 4.

Table 4. Entrapment: Gaussian probabilities.

r	$r\, N_0$	$p(N > r\, N_0)$
1	32	50%
1.41	44.6	1%
2	64	10^{-6}%
2.4	76.8	10^{-13}%

The Gaussian distribution foresees that the entire vesicle population (99%) should have $r \leq 1.41$; in other words, local intravesicle concentration factors r higher than 1.41 should occur in 1% of cases. The probability of finding vesicles with an internal concentration that is more than two times the expected one ($r > 2$) is instead vanishingly small (*ca.* 10^{-6}%), and for the case $r > 2.4$ the value becomes essentially nil (10^{-13}%). On the contrary, we have observed vesicles having r = 2–4 much more often, *i.e.*, with a frequency of about 0.1%–1%. Even if our estimations of super-filled vesicle abundance were wrong by one, two, or even three orders of magnitude, the difference between the statistical expectations and the observations would be still significant. If this is true in the worst case (C_0 = 0.1 μM), then we can be sure that it is certainly true for higher solutes concentrations (C_0 = 1–10 μM).

In conclusion, this simple statistical analysis confirms that the experimental data presented in this work again represent a conundrum for the physics of solute entrapment, because these "special" vesicles are far more abundant than what is predicted by a random encapsulation. As we suggest in previous work [9–15], a special mechanism should play a role in generating such intriguing structures.

4. Coupled Enzymatic Systems of Interest for Further Investigations

We have demonstrated that simple enzymatic systems can take advantage of micro-compartmentation being concentrated in the aqueous lumen of lipid vesicles. Although this phenomenon occurs in a limited number of vesicles, it is quite interesting because it allows the onset of efficient cell-like reactive systems starting from a diluted solution. However, model systems composed by a single reaction just provide a basic proof of concept, as it was the case of models based on inert proteins (*i.e.*, ferritin encapsulation experiments [10]). On the other hand, we have already investigated the encapsulation of complex systems like the TX-TL reaction mix, showing that the super-concentration phenomenon determines the success of otherwise difficult process, like the synthesis of proteins starting from diluted solutions. Contrary to the simplest cases, the co-encapsulation of several dozens macromolecules challenges the investigation of encapsulation statistics, hampering the construction of accurate theoretical models. In the spectrum of possible case studies, *reactive* systems of intermediate complexity can be advantageous here because they would allow a more precise analysis of co-encapsulation and at the same time function as realistic models of primitive reactive compartments.

Coupled multi-enzymatic systems can be a practical way for proceeding in the above direction. We have already designed some systems—whose reaction progress can be followed by fluorescence microscopy—that readily extend the results presented in this paper, and that are currently under investigation in our laboratory.

The strategy is based on the production of a fluorescent molecule downstream of a multi-step enzymatic pathway. In this respect, two enzymes are particularly useful, *i.e.*, peroxidase and diaphorase (*i.e.*, a NAD(P)H dehydrogenase). Peroxidase uses hydrogen peroxide for oxidizing fluorogenic substrates such as reduced fluorescein (fluorescin), reduced rhodamine, or the so-called Amplex Red© (a phenoxazine derivative) to give, respectively, fluorescein, rhodamine, and resorufin. Diaphorase uses NAD(P)H for reducing resazurin to resorufin. The activity of these two enzymes can be combined with other reactions in order to build fluorescence-detectable enzyme-catalyzed mini-pathways to be reconstructed inside vesicles. Figure 15 shows just some examples of such possible systems.

Clearly, several practical issues must be assessed for finding the best model system that can be effectively used in vesicle systems, first of all the permeability issue for all substrates, the distance from optimal environmental conditions (e.g., pH) for each enzyme, and the possibility of inferring the local enzyme concentration from setting proper experimental parameters and from observables. Numerical simulations can be helpful both for design and analysis.

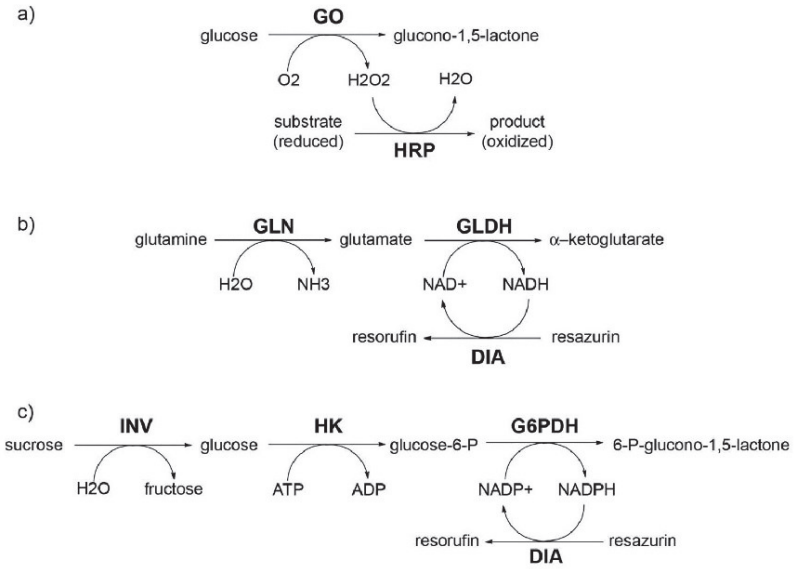

Figure 15. Examples of mini-enzymatic pathways (2, 3, and 4 steps, respectively) that could be constructed inside lipid vesicles. Abbreviations: GO: glucose oxidase; HRP: horseradish peroxidase; GLN: glutaminase; GLDH: glutamate dehydrogenase; DIA: diaphorase; INV: invertase; HK: hexokinase; G6PDH: glucose-6-phosphate dehydrogenase.

5. Concluding Remarks

In this paper we have firstly reviewed the previous results on protein and ribosome encapsulation inside vesicles, then presented new data. In contrast to previous studies, which have been carried out on conventional vesicles visualized by cryo-TEM, the present paper deals with 1–2 μm-sized vesicles and confocal fluorescence microscopy analysis. We have reported that macromolecules like proteins and dextrans can be over-encapsulated in micrometer-sized vesicles reaching a local intravesicle concentration of about 2–4 times higher than the expected value (and occasionally up to 10 times higher). Even if these enhancement factors appear to be low, they overcome the theoretical expectations, based on the Poisson (or Gaussian) distribution. As happened in previous study, the over-concentration phenomenon concerns only a small vesicle subpopulation (<1%). Moreover, in this study we have also included "dynamic" system, e.g., enzymatic systems, to show how the spontaneous over-concentration of solutes inside vesicles implies an enhanced reactivity of intra-vesicle milieu when compared with the external environment.

In addition to the obvious general—and not yet properly emphasized—message that vesicle populations are heterogeneous in terms of solute content (for a discussion, see also [53]), the main conclusion from this study is that particular physico-chemical conditions operate in a way to promote the formation of vesicles super-filled of solutes (where, by "super-filled", we mean filled with a number of solute that exceed the expected number also keeping into account the stochastic fluctuations).

We maintain that such phenomenology might have had a role in promoting the origin of early functional cells, by accumulating bioactive materials inside vesicle lumen so that reactions in confined

microenvironment could occur much better than in the external (bulk) phase. Moreover, this view emphasize the active role of lipid compartments in favoring the onset of metabolism, in the sense that lipid compartments, which form spontaneously in solution, not only provide a protected microenvironment for early reactions, and at the same time equip the compartment with a semi-permeable membrane, but also concur to the encapsulation of solutes and therefore to the very mechanism of protocell formation from separated components. Despite the fact that the super-filled vesicles represent only a small fraction of the entire population, their absolute number is nevertheless remarkable (e.g., 0.1% of a "diluted" vesicle sample, e.g., [lipids] = 1 μM, consists of *ca.* 10^5 micrometer-sized vesicle/mL).

Among the possible open questions and future directions related to this research, in addition to the investigation on the generative mechanism—also related to the power-law profiles [10,11]—the issue of nucleic acid entrapment plays a central role, especially if non-phospholipids compartments are considered, like fatty acids vesicles. Our preliminary experiments are very promising (D'Aguanno *et al.*, manuscript in preparation). It is worth mentioning that not only vesicles composed by pure compounds can be used (e.g., pure oleate vesicles), but it would be interesting to explore the behavior of lipid mixtures. A realistic scenario for primitive membranes indeed includes mixture of simple amphiphiles, with different chain length and head groups. As revealed by recent studies, such membranes might show intriguing features [54–56], but the issue of solute encapsulation has not been faced yet. Another interesting direction deals with the abovementioned reconstruction of multi-step pathways (model systems shown in Figure 15), and of exploiting the super-concentration effect to assemble high-order supramolecular structures inside lipid compartments.

6. Experimental Section

Materials. Commercially available products have been used, in particular, (*a*) from Sigma-Aldrich: dextran conjugated with rhodamine (dextran-RITC, #R8881, MW *ca.* 10 kDa), *R*-phycoerythrin (PE, #52412, MW *ca.* 240 kDa), bovine serum albumine conjugated with fluorescein (BSA-FITC, #A9771, MW *ca.* 66 kDa), allophycocyanin (APC, #A7472, MW *ca.* 104 kDa), dextran conjugated with fluorescein (dextran-FITC, #FD-150S, MW *ca.* 150 kDa), carbonic anhydrase (CA, #C3934, MW *ca.* 29 kDa), 6-carboxyfluorescein diacetate (CFDA, #C5041, 460 Da), 6-carboxyfluorescein (6-CF, #C0662, 376 Da), calceina disodium salt (#21030, 666 Da), oleic acid (#O1008), sodium oleate (#O7501), and all solvents and buffers; (*b*) from Invitrogen: proteinase K (MW *ca.* 40 kDa); (*c*) from Avanti Polar Lipids: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol (POPG) 1,2-dioleoyl-sn-glycero-3-phosphatidyl ethanolamine-lissamine-rhodamine (DOPE-lissamine-rhodamine). The block copolymer poly (butadiene-b-ethylene oxide) (PB33PEO29), MW *ca.* 1.85 kDa, was a generous gift from Prof. Stephan Förster (Bayreuth University, Germany).

Methods. Lipid vesicles were prepared by three methods: film hydration method (M1); rehydration of pre-formed extruded and freeze-dried vesicles (M2); and ethanol injection method (M3). Details of the three methods can be found in the Supplementary File of reference [14]; here we summarize only the essential points.

Film hydration method (M1). Lipid-covered glass beads (diam. 2 mm) were prepared by the evaporation method. In particular, the lipid mixture (POPC plus other lipids such as POPG or oleic

acid or DOPE-lissamine-rhodamine) was dissolved in chloroform and placed in a round-bottomed flask. Glass beads (measured by weight) were added and the solvent was removed at reduced pressure. Lipid-covered glass beads were further dried at high vacuum. The average amount of lipid/bead, measured by the Stewart assay (see [14] for details), was 10 ± 1 $nmol_{lipids}$/bead. Vesicles were obtained by hydrating the so-prepared beads with a solute-containing aqueous buffer. For example, 10 beads (100 $nmol_{lipids}$) were hydrated with 200 μL solution to obtain vesicles with a lipid concentration of 0.5 mM. Care should be taken to re-suspend effectively the lipids by continuous pipetting up and down the aqueous solution. Note that essentially similar results have been obtained when the lipid film was directly prepared in a Eppendorf-like tube, starting from lipid solution in methanol, and next evaporation of the solvent by a centrifugal evaporator (e.g., Savant SpeedVac® or similar).

Rehydration of extruded and freeze-dried vesicles (M2). POPC vesicles were first prepared by the thin film hydration method. Vesicles were extruded by 10 passages over two stacked Whatman Nuclepore Track-Etch polycarbonate membranes mounted on a hand-extruder (Avestin Liposofast) with pore size 400 or 800 nm. The so-obtained extruded vesicles were freeze-dried so to obtain a vesicle "cake" that was then hydrated with an appropriate amount of solute-containing solution. The final POPC concentration was 2.5–5 mM.

Ethanol injection method (M3). An ethanol solution of lipids (100 mM) was injected, by means of a Hamilton microsyringe or a Gilson micropipette, in the solution containing the solute(s) of interest, in a 1:30 to 1:60 volume ratio, so that the final lipid concentration was 1.7–3.3 mM and the final ethanol amount was 1.7%–3.3% v/v. The lipids used were: (*i*) POPC; (*ii*) POPC/sodium oleate 4/1 mol/mol. In the latter case, the stock solution was prepared from POPC/ethanol and sodium oleate/methanol stock solutions.

Preliminary experiments were carried out to select the best buffer. 50–200 mM HEPES (pH 7.4) as well as phosphate-buffered saline (PBS, pH 7.4) produced mostly vesicle aggregates. Phosphate buffers (pH 7) produced vesicle aggregates at high phosphate concentration (200–400 mM), and good vesicle (both for POPC or POPC:oleate 4:1 mol/mol) samples at low phosphate concentration (50–100 mM). Britton-Robinson buffer (pH 7) gave different results depending on the lipids used.

The above mentioned methods have been used to prepare solute-containing vesicles, in particular, the following solution have been used: BSA-FITC (0.63–5 μM), dextran-FITC (0.63–5 μM), dextran-RITC (0.63–5 μM), PE (0.16–0.25 μM), APC (0.5–5 μM), CA (0.25–1 μM), proteinase K plus BSA-FITC (1 μM plus 2.5–5 μM, respectively). The buffer was 100 mM sodium phosphate (pH 7) if not specified otherwise (see Tables 1–3 footnotes). The non-encapsulated solutes were never removed.

Carbonic anhydrase (CA) plus carboxyfluorescein diacetate (CFDA) reaction was carried out as it follows. First, CA-containing vesicles were prepared by one of the above-described methods. Next, CFDA was added from a stock acetonitrile solution (the final acetonitrile content was ≤5 vol%). After CFDA addition (<20 s), the sample was quickly transferred in the glass chamber and imaged by confocal microscopy.

Proteinase K plus BSA-FITC reaction was carried out by forming vesicles in a freshly prepared solution which contained proteinase K (1 μM) plus BSA-FITC (2.5–5 μM). After vesicle formation (<1 min), the sample was quickly transferred in the glass chamber and imaged by confocal microscopy.

Glassware preparation for microscopy analysis. All glassware was cleaned with ethanol and lint-free paper (Kimwipes Lite, Kimberly-Clark) to remove dust and contaminants from the surface.

The observation chamber was homemade by creating a properly shaped spacer with two parafilm layers placed between the glass slide and the cover slip. This three-layer construction was placed over a heater plate to melt the parafilm. In each "chamber" about 20–40 μL of each solution can be placed.

Confocal microscopy analysis was carried out by using (*i*) Zeiss LSM 510 inverted microscope or (*ii*) Leica TCS SP5 inverted microscope. Laser power, gain, offsets were optimized for each experiment in order not to saturate the detector. This was particularly important in kinetic experiments, where fluorescence increases in time. Calibration lines were first constructed by using increasing amount of solutes (dextran-RITC, dextran-FITC, BSA-FITC, APC, PE, 6-carboxyfluorescein), in order to convert fluorescence units (8-bits values, from 0 to 255) in molar concentration. In no cases we observed self-quenching or non-linear responses, also thanks to the small sample thickness. Preliminary experiments were carried out by recording the fluorescence profile in *z* direction in order to determine the most suitable depth of focal plane (generally occurring at >5 μm from the surface), to avoid surface scattering and other surface-related phenomena. The fluorescence intensity of the vesicles and of the background was quantified by image analysis carried out by ImageJ software (http://rsbweb.nih.gov/ij/) [45].

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Author Contributions

The question on the mechanisms of macromolecules entrapment inside lipid vesicles was prompted by Pier Luigi Luisi at the Roma Tre University (Rome, Italy), and started on this subject a specific research program in collaboration with Alfred Fahr (Friedrich-Schiller-Universität Jena, Germany). Erica D'Aguanno and Emiliano Altamura carried out the experiments. All authors devised the experiments and wrote the paper. All authors have read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Ruiz-Mirazo, K.; Briones, C.; de la Escosura, A. Prebiotic Systems Chemistry: New Perspectives for the Origins of Life. *Chem. Rev.* **2014**, *114*, 285–366.

2. Luisi, P.L. *The Emergence of Life: From Chemical Origins to Synthetic Biology*, 1st ed.; Cambridge University Press: Cambridge, UK, 2006.
3. Luisi, P.L.; Ferri, F.; Stano, P. Approaches to Semi-Synthetic Minimal Cells: A Review. *Naturwissenschaften* **2006**, *93*, 1–13.
4. Berclaz, N.; Muller, M.; Walde, P.; Luisi, P.L. Growth and Transformation of Vesicles Studied by Ferritin Labeling and Cryotransmission Electron Microscopy. *J. Phys. Chem. B* **2001**, *105*, 1056–1064.
5. Berclaz, N.; Blochliger, E.; Muller, M.; Luisi, P.L. Matrix Effect of Vesicle Formation as Investigated by Cryotransmission Electron Microscopy. *J. Phys. Chem. B* **2001**, *105*, 1065–1071.
6. Oberholzer, T.; Nierhaus, K.H.; Luisi, P.L. Protein Expression in Liposomes. *Biochem. Biophys. Res. Commun.* **1999**, *261*, 238–241.
7. Oberholzer, T.; Luisi, P.L. The Use of Liposomes for Constructing Cell Models. *J. Biol. Phys.* **2002**, *28*, 733–744.
8. Murtas, G.; Kuruma, Y.; Bianchini, P.; Diaspro, A.; Luisi, P.L. Protein Synthesis in Liposomes with a Minimal Set of Enzymes. *Biochem. Biophys. Res. Commun.* **2007**, *363*, 12–17.
9. Souza, T.P.; Stano, P.; Luisi, P.L. The Minimal Size of Liposome-Based Model Cells Brings about a Remarkably Enhanced Entrapment and Protein Synthesis. *Chembiochem Eur. J. Chem. Biol.* **2009**, *10*, 1056–1063.
10. Luisi, P.L.; Allegretti, M.; de Souza, T.P.; Steiniger, F.; Fahr, A.; Stano, P. Spontaneous Protein Crowding in Liposomes: A New Vista for the Origin of Cellular Metabolism. *Chembiochem* **2010**, *11*, 1989–1992.
11. Souza, T.P.; Steiniger, F.; Stano, P.; Fahr, A.; Luisi, P.L. Spontaneous Crowding of Ribosomes and Proteins inside Vesicles: A Possible Mechanism for the Origin of Cell Metabolism. *Chembiochem* **2011**, *12*, 2325–2330.
12. Souza, T.P.; Stano, P.; Steiniger, F.; D'Aguanno, E.; Altamura, E.; Fahr, A.; Luisi, P.L. Encapsulation of Ferritin, Ribosomes, and Ribo-Peptidic Complexes Inside Liposomes: Insights Into the Origin of Metabolism. *Orig. Life Evol. Biospheres* **2012**, *42*, 421–428.
13. Luisi, P.L. An Open Question on the Origin of Life: The First Forms of Metabolism. *Chem. Biodivers.* **2012**, *9*, 2635–2647.
14. Stano, P.; D'Aguanno, E.; Bolz, J.; Fahr, A.; Luisi, P.L. A Remarkable Self-Organization Process as the Origin of Primitive Functional Cells. *Angew. Chem. Int. Ed.* **2013**, *52*, 13397–13400.
15. De Souza, T.P.; Fahr, A.; Luisi, P.L.; Stano, P. Spontaneous Encapsulation and Concentration of Biological Macromolecules in Liposomes: An Intriguing Phenomenon and Its Relevance in Origins of Life. *J. Mol. Evol.* **2014**, *79*, 179–192.
16. Kruger, K.; Grabowski, P.J.; Zaug, A.J.; Sands, J.; Gottschling, D.E.; Cech, T.R. Self-Splicing RNA: Autoexcision and Autocyclization of the Ribosomal RNA Intervening Sequence of Tetrahymena. *Cell* **1982**, *31*, 147–157.
17. Zhang, B.L.; Cech, T.R. Peptidyl-Transferase Ribozymes: Trans Reactions, Structural Characterization and Ribosomal RNA-like Features. *Chem. Biol.* **1998**, *5*, 539–553.
18. Joyce, G.F. Building the RNA World. Ribozymes. *Curr. Biol. CB* **1996**, *6*, 965–967.
19. Szostak, J.W.; Bartel, D.P.; Luisi, P.L. Synthesizing Life. *Nature* **2001**, *409*, 387–390.

20. Mavelli, F. Stochastic Simulations of Minimal Cells: The Ribocell Model. *BMC Bioinform.* **2012**, *13*, doi:10.1186/1471-2105-13-S4-S10.
21. Stano, P.; Carrara, P.; Kuruma, Y.; de Souza, T.P.; Luisi, P.L. Compartmentalized Reactions as a Case of Soft-Matter Biotechnology: Synthesis of Proteins and Nucleic Acids inside Lipid Vesicles. *J. Mater. Chem.* **2011**, *21*, 18887–18902.
22. De Lorenzo, V.; Danchin, A. Synthetic Biology: Discovering New Worlds and New Words. *EMBO Rep.* **2008**, *9*, 822–827.
23. Chiarabelli, C.; Stano, P.; Luisi, P.L. Chemical approaches to synthetic biology. *Curr. Opin. Biotech.* **2009**, *20*, 492–497.
24. Gil, R.; Silva, F.J.; Peretó, J.; Moya, A. Determination of the Core of a Minimal Bacterial Gene Set. *Microbiol. Mol. Biol. Rev. MMBR* **2004**, *68*, 518–537.
25. Moya, A.; Gil, R.; Latorre, A.; Peretó, J.; Pilar Garcillán-Barcia, M.; de la Cruz, F. Toward Minimal Bacterial Cells: Evolution *vs.* Design. *FEMS Microbiol. Rev.* **2009**, *33*, 225–235.
26. Shimizu, Y.; Inoue, A.; Tomari, Y.; Suzuki, T.; Yokogawa, T.; Nishikawa, K.; Ueda, T. Cell-Free Translation Reconstituted with Purified Components. *Nat. Biotechnol.* **2001**, *19*, 751–755.
27. Shimizu, Y.; Kanamori, T.; Ueda, T. Protein Synthesis by Pure Translation Systems. *Methods* **2005**, *36*, 299–304.
28. Lasic, D.D. The Mechanism of Vesicle Formation. *Biochem. J.* **1988**, *256*, 1–11.
29. Van Hoof, B.; Markvoort, A.J.; van Santen, R.A.; Hilbers, P.A.J. On Protein Crowding and Bilayer Bulging in Spontaneous Vesicle Formation. *J. Phys. Chem. B* **2012**, *116*, 12677–12683.
30. Van Hoof, B.; Markvoort, A.J.; van Santen, R.A.; Hilbers, P.A.J. Molecular Simulation of Protein Encapsulation in Vesicle Formation. *J. Phys. Chem. B* **2014**, *118*, 3346–3354.
31. Walde, P. Surfactant Assemblies and Their Various Possible Roles for the Origin(S) of Life. *Orig. Life Evol. Biosph.* **2006**, *36*, 109–150.
32. Matthäus, C.; Bird, B.; Miljković, M.; Chernenko, T.; Romeo, M.; Diem, M. Infrared and Raman Microscopy in Cell Biology. *Methods Cell Biol.* **2008**, *89*, 275–308.
33. Nishimura, K.; Hosoi, T.; Sunami, T.; Toyota, T.; Fujinami, M.; Oguma, K.; Matsuura, T.; Suzuki, H.; Yomo, T. Population Analysis of Structural Properties of Giant Liposomes by Flow Cytometry. *Langmuir ACS J. Surf. Colloids* **2009**, *25*, 10439–10443.
34. Nishimura, K.; Matsuura, T.; Nishimura, K.; Sunami, T.; Suzuki, H.; Yomo, T. Cell-Free Protein Synthesis inside Giant Unilamellar Vesicles Analyzed by Flow Cytometry. *Langmuir ACS J. Surf. Colloids* **2012**, *28*, 8426–8432.
35. Dominak, L.M.; Keating, C.D. Polymer Encapsulation within Giant Lipid Vesicles. *Langmuir* **2007**, *23*, 7148–7154.
36. Bangham, A.D.; Standish, M.M.; Watkins, J.C. Diffusion of Univalent Ions across the Lamellae of Swollen Phospholipids. *J. Mol. Biol.* **1965**, *13*, 238–252.
37. Nourian, Z.; Roelofsen, W.; Danelon, C. Triggered Gene Expression in Fed-Vesicle Microreactors with a Multifunctional Membrane. *Angew. Chem. Int. Ed. Engl.* **2012**, *51*, 3114–3118.
38. Shew, R.L.; Deamer, D.W. A Novel Method for Encapsulation of Macromolecules in Liposomes. *Biochim. Biophys. Acta* **1985**, *816*, 1–8.
39. Ishikawa, K.; Sato, K.; Shima, Y.; Urabe, I.; Yomo, T. Expression of a Cascading Genetic Network within Liposomes. *FEBS Lett.* **2004**, *576*, 387–390.

40. Batzri, S.; Korn, E.D. Single Bilayer Liposomes Prepared without Sonication. *Biochim. Biophys. Acta BBA—Biomembr.* **1973**, *298*, 1015–1019.
41. Domazou, A.S.; Luisi, P.L. Size Distribution of Spontaneously Formed Liposomes by the Alcohol Injection Method. *J. Liposome Res.* **2002**, *12*, 205–220.
42. Stano, P.; Bufali, S.; Pisano, C.; Bucci, F.; Barbarino, M.; Santaniello, M.; Carminati, P.; Luisi, P.L. Novel Camptothecin Analogue (gimatecan)-Containing Liposomes Prepared by the Ethanol Injection Method. *J. Liposome Res.* **2004**, *14*, 87–109.
43. Tashian, R.E.; Douglas, D.P.; Yu, Y.S. Esterase and hydrase activity of carbonic anhydrase. I. From primate erythrocytes. *Biochem. Biophys. Res. Commun.* **1964**, *14*, 256–261.
44. Verpoorte, J.A.; Mehta, S.; Edsall, J.T. Esterase activities of human carbonic anhydrases B and C. *J. Biol. Chem.* **1967**, *242*, 4221–4229.
45. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **2012**, *9*, 671–675.
46. Thomae, A.V. Experimental and Theoretical Investigations on Lipid Bilayer Permeation. Ph.D. Thesis, Eidgenössische Technische Hochschule ETH, Zurich, Switzerland, 2007.
47. New, R.R.C. *Liposomes. A Practical Approach*, 1st ed.; IRL Press at Oxford University Press: Oxford, UK, 1990.
48. Hoops, S.; Sahle, S.; Gauges, R.; Lee, C.; Pahle, J.; Simus, N.; Singhal, M.; Xu, L.; Mendes, P.; Kummer, U. COPASI—a COmplex PAthway SImulator. *Bioinformatics* **2006**, *22*, 3067–3074.
49. De Lumen, B.O.; Tappel, A.L. Fluorescein-hemoglobin as a substrate for cathepsin D and other proteases. *Anal. Biochem.* **1970**, *36*, 22–29.
50. Voss, E.W., Jr.; Workman, C.J.; Mummert, M.E. Detection of Protease Activity Using a Fluorescence-Enhancement Globular Substrate. *BioTechniques* **1996**, *20*, 286–291.
51. Kristjánsson, M.M.; Magnússon, Ó.T.; Gudmundsson, H.M.; Alfredsson, G.Á.; Matsuzawa, H. Properties of a subtilisin-like proteinase from a psychrotrophic Vibrio species. Comparison with proteinase K and aqualysin I. *Eur. J. Biochem.* **1999**, *260*, 752–760.
52. Georgieva, D.; Genov, N.; Voelter, W.; Betzel, C. Catalytic Efficiencies of Alkaline Proteinases from Microorganisms. *Z. Naturforsch C* **2006**, *61*, 445–452.
53. Stano, P.; Souza, T.; Carrara, P.; Altamura, E.; D'Aguanno, E.; Caputo, M.; Luisi, P.L.; Mavelli, F. Recent biophysical issues about the preparation of solute-filled lipid vesicles. *Mech. Adv. Mat. Struct.* **2015**, doi: 10.1080/15376494.2013.857743.
54. Mansy, S.S.; Schrum, J.P.; Krishnamurthy, M.; Tobé, S.; Treco, D.A.; Szostak, J.W. Template-directed synthesis of a genetic polymer in a model protocell. *Nature* **2008**, *454*, 122–125.
55. Maurer, S.E.; Deamer, D.W.; Boncella, J.M.; Monnard, P.A. Chemical evolution of amphiphiles: Glycerol monoacyl derivatives stabilize plausible prebiotic membranes. *Astrobiology* **2009**, *9*, 979–987.
56. Budin, I.; Prywes, N.; Zhang, N.; Szostak, J.W. Chain-length heterogeneity allows for the assembly of fatty acid vesicles in dilute solutions. *Biophys. J.* **2014**, *107*, 1582–1590.

3.1 Publication 2

A remarkable self-organization process as the origin of primitive functional cells.

Pasquale Stano, Erica D'Aguanno , Jürgen Bolz , Alfred Fahr , Pier Luigi Luisi

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Synthetic Biology Hot Paper

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A Remarkable Self-Organization Process as the Origin of Primitive Functional Cells**

*Pasquale Stano, Erica D'Aguanno, Jürgen Bolz, Alfred Fahr, and Pier Luigi Luisi**

Research into the origin of life has often focused on the chemistry of self-replication,[1–3] whereas less attention has been devoted to experiments that might reveal the emergence of cells as compartmentalized and dynamic chemical systems. Modern views consider lipid vesicles (liposomes) as the most plausible model of primitive cellular systems;[4–6] however, experimental investigations into liposome formation and solute entrapment, a key event for the origin of cells, has not lead to an explanation for the assembly of a functional genetic–metabolic network inside liposomes to date.

One way to tackle the question about the origin of cells is to carry out experimental research on simplified chemical systems. Such models should be complex enough so as to display a simple-cell-like behavior; however, they should be well-characterized in terms of composition and function.

As a viable model for investigating the onset of "minimal" metabolism inside liposomes, we investigated protein synthesis by cell-free transcription and translation (TX-TL) systems. These multimolecular systems contain a total of about 80 different macromolecular components, including RNA polymerase, ribosomes, tRNAs, aminoacyl-tRNA synthases, translation factors, and bioenergy-related enzymes (as well as low-molecular-weight compounds such as amino acids and nucleotides), and are capable of synthesizing a protein starting from the corresponding DNA sequence.

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In a TX-TL system, such as a commercially available *E. coli* cellular extract or the well-characterized PURE system[7] (a reconstituted kit that contains the minimal number of molecules necessary for TX-TL activity), a functional protein is produced as soon as the corresponding DNA sequence is supplied to the mixture. This happens because the commercial TX-TL mixture is concentrated, as it comes directly from a cell extract. The real question then is, how such a critical concentration of the components might have been reached under prebiotic conditions. In fact, when we consider a prebiotic solution and assume that the macromolecular components have somehow developed by themselves, the solution in a primitive marine or lagoon environment can only be highly diluted and therefore unreactive.

We reasoned that such an accumulation of components could occur within liposomes, which might thus provide an active role in concentrating the TX-TL molecules, as we reported for a simple case of single-species entrapment (ferritin[8] or ribosomes[9] were used as model compounds). In other words, we propose a scenario where the formation and closure of lipid membranes to form liposomes could have been the driving force for a high local concentration (overcrowding) of diluted solutes, an event that, in principle, may have led to the onset of metabolism in primitive cells.

Herein, we therefore focus on the investigation of the synthesis of proteins inside liposomes, which are formed in situ in the presence of a diluted TX-TL mixture. Such a dilute, non-reactive solution simulates a possible origin-of-life scenario in a marine or fresh-water lagoon, where the components of life (DNA, RNA, and proteins) have formed independently, but cannot react because of the extremely high dilution. In particular, we address the question whether the spontaneous formation of liposomes might bring about a spontaneous concentration of all components of the mixture inside vesicles, so that protein synthesis proceeds efficiently within these cell-like compartments, whereas the same reaction does not proceed in free solution (Figure 1).

The original TX-TL mixture already becomes unreactive when it is diluted with a buffer in a 1:1 ratio (for details, see the Supporting Information). A small aliquot of a phosphatidylcholine–ethanol solution is now injected into this dilute and unreactive TX-TL mixture to form liposomes of various size and morphology by spontaneous self-assembly. In particular, we used 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), a well-characterized phospholipid that has been extensively used in similar protein-synthesis experiments, because of its stability and minimal interference with TX-TL reaction, as well as its low melting temperature (ca. −4 °C). POPC vesicles entrap the components of the TX-TL mixture during their formation, whereby the macro-

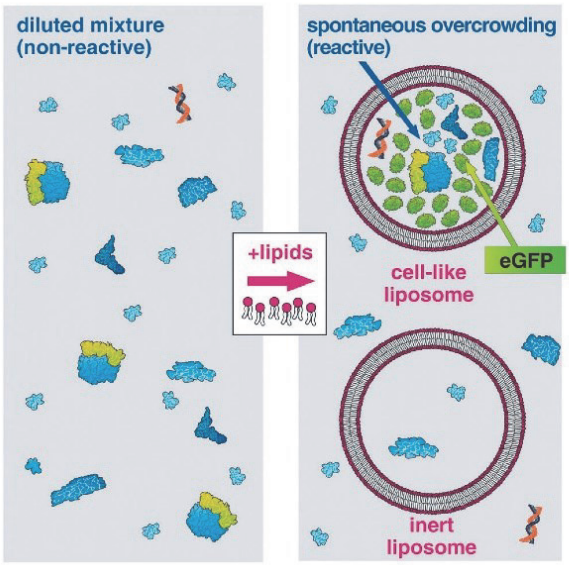

Figure 1. An experimental model for the origin of cellular metabolism. Lipid vesicles (liposomes) are formed in a diluted, sluggishly reacting (or unreactive) functional macromolecular mixture that simulates diluted primitive aqueous solutions. Because of lipid self-assembly, liposomes form spontaneously in this solution and entrap, also spontaneously, the available solutes, which gives rise to "protocells". Under these diluted conditions, the formation of inactive compartments is expected. A small but measurable number of solute-filled liposomes form spontaneously against the statistical expectation, which gives rise to cell-like systems where the high local (intraliposome) solute concentration triggers the onset of protocell metabolism. The transcription–translation (TX-TL) biomolecular machinery was taken as a model of complex cellular function.

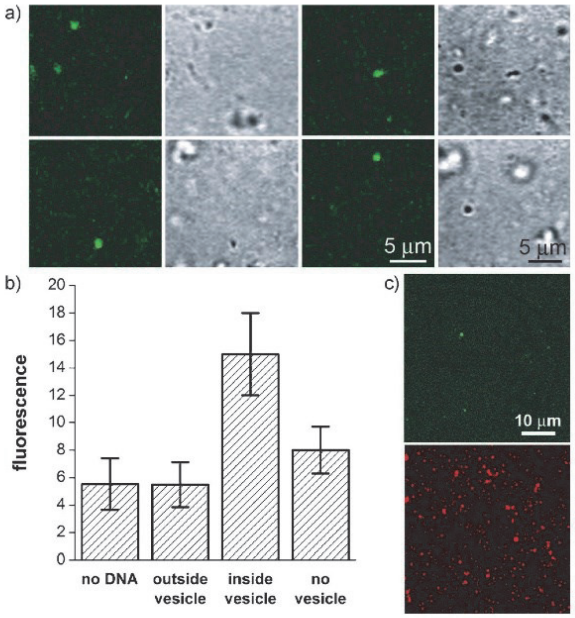

Figure 2. Liposomes that synthesize eGFP are prepared by the ethanol injection method. a) Confocal microscopy images of POPC liposomes (3.3 mM) prepared by the ethanol injection method in the presence of a diluted PURE system solution (×0.65). Bright vesicles are visible against a dark background, which indicates intraliposomal eGFP synthesis. Images have been modified for higher contrast (see the Supporting Information for details). Additional images of fluorescent liposomes that were prepared by other methods, and in the presence of cell extracts and different dilution factors are presented in Figure S6. b) Quantitation of eGFP fluorescence in negative-control samples (no DNA), in samples of the bulk solutions (extra-liposome), in liposomes, and in the absence of liposomes. c) Nile-Red staining (bottom panel) of eGFP producing liposomes (top panel) reveals that the large majority of liposomes do not synthesize the protein.

molecular species cannot escape through the POPC bilayer because of their large size. Similarly, non-entrapped macromolecules cannot enter the vesicle. We incubated the sample for 2–4 h at 37 °C to allow the synthesis of enhanced green fluorescent proteins (eGFPs) inside and outside vesicles. Aside from small vesicles, the sample contained large vesicles, the size of which generally ranges from approximately 0.7–2 µm, which could be well visualized by confocal microscopy.

Typical images of the vesicles in the sample are shown in Figure 2a. Green fluorescent vesicles are clearly visible against a dark background, which indicates that eGFP is effectively produced inside the vesicles, whereas the same reaction does not proceed at a measurable rate in the free solution. In control experiments, we confirmed the fluorescence versus concentration profiles to be linear. Deviations of the inner filter effect are negligible because of the short path length of the specimen (< 50 µm). A quantitative comparison of the external fluorescence with a negative control sample (a similarly diluted TX-TL mixture prepared in the absence of DNA) confirmed that eGFP is not produced outside the vesicles, or, strictly speaking, that its concentration remains below the detection limit (Supporting Information, Figure S1). The resulting structure, a liposome compartment containing the biochemical machinery most important for life, can be seen as good model for primitive cells that form spontaneously by a self-organization process. In other words, the mechanism of vesicle self-assembly might promote the spontaneous concentration of solutes within these microcompartments. This allows and triggers biochemical reactions that would not occur in diluted solutions. The on/off behavior that follows from the remarkable enhancement in the yield of protein synthesis that is induced by the high concentration of solutes inside liposomes is clearly shown in Figure 2b. The presence of POPC liposomes hardly affects the eGFP synthesis.

The number of green fluorescent vesicles (eGFP synthesizing) within the sample was quite low. To estimate the fraction of green fluorescent vesicles within the vesicle population, we stained the vesicles with a red-fluorescent dye (Nile Red), as shown in Figure 2c. Image analysis reveals that less than approximately 0.5% of the vesicles display an overcrowding of the components of the TX-TL kit. This percentage, even if low, sharply contrasts with the statistical expectation value, which is essentially zero (Supporting Information, Table S1). The probability of co-entrapping 83 different macromolecules (the TX-TL components of the PURE system), considered as a product of independent

events, is our null-hypothesis against which we contrasted our experimental data. As it is expected that the TX-TL components interact dynamically with each other, and perhaps form hyperstructures,[10,11] we have also calculated the co-entrapment probability for 40 or 20 different macromolecular complexes that originate from macromolecular associations within the TX-TL kit. The expected values would not explain the observations under any circumstances. Expected probabilities become similar to experimentally observed frequencies only if few and very large complexes between the TX-TL components are considered (such as five or ten large complexes).

Alternatively, in a scenario where protein synthesis would depend on the concentration of only a few key components, spontaneous two-fold concentration of these components could also explain the data. Poisson statistics suggest that components that are present at the lowest concentration could play such role (for example, DNA), or components, whose activity is critical for the success of protein synthesis (for example, ribosomes).

On the other hand, the amount of eGFP-producing vesicles (0.5%) found is similar to the amount of super-filled vesicles found in the case of ferritin and ribosomes entrapment,[8,9] which suggests that the encapsulation of the multicomponent TX-TL kit could also follow a power-law distribution (as for ferritin and ribosomes), rather than a Poisson distribution. It is clear that further evidence is required to establish what is the best suitable statistical model for a comparison with the experimental data.

In comparative experiments, POPC vesicles were formed by the hydration of thin films deposited on glass beads (2 mm), with or without an extrusion step (800 nm). Similar results were observed in all cases, both for *E. coli* cell extracts and the PURE system. The full set of experimental conditions and typical results can be found in the Supporting Information. Aside from POPC, vesicles were also prepared from a sodium oleate/POPC mixture in a 1:4 ratio by the injection method. A discussion on the effect of experimental variables and on the relation between vesicle size and internal fluorescence is given in the Supporting Information (Table S2 and Figure S2).

We also checked the spectral features of eGFP fluorescence inside vesicles to further strengthen our conclusions about its intravesicle production. The emission spectrum obtained by microspectrofluorimetry of eGFP produced in bulk solution is shown in Figure S3a, and it is compared with the spectrum recorded by a fluorimeter; these show perfect similarity. The eGFP emission spectrum can be deconvoluted as a sum of two Gaussian emission bands, centered at 511 and 531 nm, respectively.[12] The ratio between the two band intensities for bulk eGFP is 1.8 ± 0.1 (Figure 3a). Whereas some green fluorescent vesicles actually show an emission spectrum that fits well with that one of eGFP in bulk solution, others display a significantly different spectrum (Figure S3b), which is characterized by a different ratio of the two band intensities. In particular, the intensity of the 511 nm band decreases, whereas the one of the 531 nm band increases, and their ratio dramatically drops to lower values, in the range of $0.9–0.5 \pm 0.1$. A typical case is shown in Figure 3b. Previous

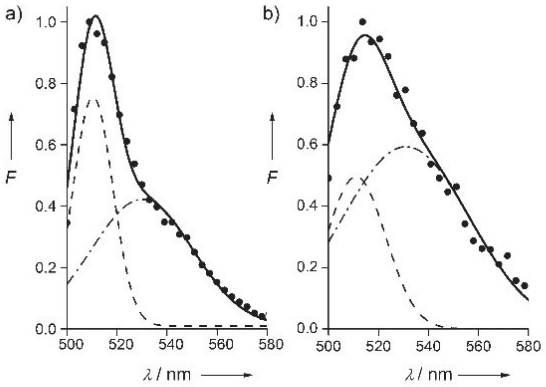

Figure 3. eGFP emission spectra recorded by microspectrofluorimetry. a) Bulk eGFP spectrum (●), b) intra-liposome eGFP spectrum (●). Each emission spectrum was fitted as a sum (——) of two Gaussian bands centered at 511 nm (– – –) and 531 nm (–•–•), respectively. The different spectral shape in (b) is due to the varied ratio between the two peaks, owing to locally concentrated eGFP.

studies have shown that closely packed eGFP molecules that were immobilized in a gel display a similar change in the emission spectrum,[13] which has been tentatively explained as energy transfer between molecules. In our case, the observation of such anomalous eGFP emission spectra inside liposomes suggests that in those vesicles that were able to over-concentrate the TX-TL solutes, the internal eGFP synthesis proceeded so well that its local concentration becomes comparable to densely packed eGFP arrays. This evidence further supports our interpretation of the data in terms of few super-active vesicles formed thanks to exceptionally favorable entrapment events. In turn, the locally concentrated TX-TL molecular machinery efficiently produces functional eGFP molecules that accumulate inside the vesicles.

We believe that the low amount of such overcrowded vesicles is a signature of the underlying specific, local, and stochastic conditions that favor their formation. The driving force could be related to the release of free water (at the expense of macromolecule surface-bound water, which also extends down to multiple layers), so that the observations could be tentatively explained as a kind of phase transition. We are currently investigating possible generative mechanisms by means of stochastic simulations.

From the viewpoint of experimental investigations, the mechanism leading to solutes of "super-concentration" inside vesicles is a conundrum, and several questions are still unanswered. A more detailed and quantitative analysis of protein encapsulation can be obtained by fluorescence-tagged proteins, possibly using several proteins to assess the statistics of co-encapsulation by confocal microscopy or a flow cytometer. Averaging techniques based on the quantitation of the overall solute encapsulation (or the overall reaction products) are not suitable in this context. A detailed entrapment analysis would be informative for moderately complex mixtures, and help to formulate a quantitative model for this intriguing phenomenon. However, a functional assay on the success of a reaction in terms of end product detection as

in the case of eGFP synthesis remains a very stringent test for real-case mixtures. The exploration of over-concentration effects in vesicles prepared by other methods, such as detergent depletion, reverse phase evaporation, and water-in-oil droplet transfer, would further expand the current available observations[8,9,14] (when the method itself does not impair the activity of the solutes of interest).

In conclusion, we have shown that the formation of functionally active primitive cell models is due to a remarkable self-organization step that involves lipids and water-soluble molecules. Because of the spontaneous concentration of the solutes inside micrometer-sized liposomes, it was possible to observe the occurrence of a cell-like complex biochemical pathway, whereas no reaction occurred in free solution. Aside from obvious routes for concentrating solutes, including the evaporation of water in lagoons, our data emphasize the role of micro-compartmentalization during the origin of life, and provide a physically realistic model for understanding the emergence of early cells. Moreover, understanding the generative mechanism for the formation of solutes of super-concentration inside vesicles could allow the development of preparative methods that facilitate their formation. This, in turn, would be of great relevance for practical applications, especially in the nano-biotechnological field.

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[1] L. E. Orgel, *Nature* **1992**, *358*, 203 – 209.
[2] J. W. Szostak, D. P. Bartel, P. L. Luisi, *Nature* **2001**, *409*, 387 – 390.
[3] T. A. Lincoln, G. F. Joyce, *Science* **2009**, *323*, 1229 – 1232.
[4] D. W. Deamer, *Nature* **1985**, *317*, 792 – 794.
[5] P. Walde, A. Goto, P. A. Monnard, M. Wessicken, P. L. Luisi, *J. Am. Chem. Soc.* **1994**, *116*, 7541 – 7547.
[6] S. S. Mansy, J. P. Schrum, M. Krishnamurthy, S. Tobé, D. A. Treco, J. W. Szostak, *Nature* **2008**, *454*, 122 – 125.
[7] Y. Shimizu, A. Inoue, Y. Tomari, T. Suzuki, T. Yokogawa, K. Nishikawa, T. Ueda, *Nat. Biotechnol.* **2001**, *19*, 751 – 755.
[8] P. L. Luisi, M. Allegretti, T. Souza, F. Steineger, A. Fahr, P. Stano, *ChemBioChem* **2010**, *11*, 1989 – 1992.
[9] T. Pereira de Souza, F. Steineger, P. Stano, A. Fahr, P. L. Luisi, *ChemBioChem* **2011**, *12*, 2325 – 2330.
[10] M. Hoppert, F. Mayer, *Cell Biochem. Biophys.* **1999**, *31*, 247 – 284.
[11] V. Norris, T. den Blaauwen, A. Cabin-Flaman, R. H. Doi, R. Harshey, L. Janniere, A. Jimenez-Sanchez, D. J. Jin, P. A. Levin, E. Mileykovskaya, A. Minsky, M. Saier, Jr., K. Skarstad, *Microbiol. Mol. Biol. Rev.* **2007**, *71*, 230 – 253.
[12] M. Chattoraj, B. A. King, G. U. Bublitz, S. G. Boxer, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 8362 – 8367.
[13] R. A. G. Cinelli, A. Ferrari, V. Pellegrini, M. Tyagi, M. Giacca, F. Beltram, *Photochem. Photobiol.* **2000**, *71*, 771 – 776.
[14] T. Souza, P. Stano, F. Steiniger, E. D'Aguanno, E. Altamura, A. Fahr, P. L. Luisi, *Origins Life Evol. Biospheres* **2012**, *42*, 421 – 428.

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Supporting Information

69451 Weinheim, Germany

A Remarkable Self-Organization Process as the Origin of Primitive Functional Cells**

*Pasquale Stano, Erica D'Aguanno, Jürgen Bolz, Alfred Fahr, and Pier Luigi Luisi**

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SUPPLEMENTARY MATERIAL | CONTENTS

A1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) was from Avanti (Alabaster, AL, USA). The plasmid pWM-T7-EGFP (3026 bp) was from Biotecton (Zürich, Switzerland). Recombinant EGFP was from BD Bioscience (Basel, Switzerland). The cell-free expression kit (#L1130, "*E. coli* T7S30 extract system for circular DNA") and the amino acid mixture (#L4461) were from Promega. The transcription–translation kit composed of purified components (PURE system) was from the laboratory of Prof. T. Ueda (University of Tokyo). 2-mm Glass beads (#104014, #104015) were from Merck. Nile Red (#72485) and all others chemicals were bought from Sigma-Aldrich.
The "dilution buffer" consisted in: (*a*) Multi-Core buffer (#R9991, Promega), i.e., 25 mM Tris acetate (pH 7.5), 100 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT; or (*b*) autoclaved and 200 nm filtered 50 mM Hepes KOH (pH 7.5), 100 mM potassium glutamate, 13 mM magnesium acetate, 2 mM spermidine, 1 mM DTT.

A2. Methods

Mixing of transcription–translation (TX-TL) elements, preparation of vesicles and vesicle manipulations were all done at 4 °C to prevent the premature start of TX-TL reactions. For preparing 100 μL of reactive, not-diluted TX-TL kits, we mixed, in the case of cell extracts (Promega): S30 extracts (50 μL), T7S30 extracts (30 μL), amino acid complete mixture (10 μL), distilled water (6 μL), and 1.3 mg/mL pWM-T7-EGFP plasmid in water (4 μL). In the case of PURE system, we mixed "A" solution (50 μL), "B" solution (20 μL), 1 mM DTT (2 μL), distilled water (26 μL), and 1.3 mg/mL pWM-T7-EGFP DNA plasmid in water (2 μL). Diluted TX-TL kits were prepared from not-diluted ones by mixing with an appropriate volume of "dilution buffer" (see Materials). (a) Ethanol injection method. A 100 mM POPC in ethanol solution was injected in the diluted TX-TL mix kept at 4 °C, to obtain liposomes at a final 1.7-3.3 mM (final ethanol 1.7-3.3 % v/v). The suspension was mixed by pipetting. (b) Thin film hydration method. POPC-covered glass beads (diameter: 2 mm) were added to 100 uL of diluted TX-TL mix, and liposomes were formed by gently vortexing and pipetting. The final POPC concentration was from 0.2 to 1.6 mM. (c) Additional experiments were carried out with extruded liposomes (diameter: 800 nm) and with liposomes obtained after a freeze-drying process (see details in the Supplementary Information). Liposome suspensions were incubated at 37 °C for 2-4 hours, then analyzed by confocal laser scanning microscopy. When necessary, liposomes were stained by Nile Red, which was added (1:5 v/v) as saturated water solution (ca. 1.7 μg/mL). Negative control experiments were done by using a TX-TL mixture deprived of pWM-T7-EGFP DNA plasmid.

Preparation, quantitation, and use of POPC-coated glass beads. Glass beads with diameter 2-3 mm are practical and gave the best results. Firstly, 500-1000 glass beads (measured by weight) were placed in a 100 mL round bottomed flask and washed twice with chloroform, then dried under vacuum. Then, POPC was added as chloroform solution and the solvent was evaporated under reduced pressure, to produce POPC-coated glass beads. These were further dried under high vacuum for 6 hours and then transferred (without touching them) in tubes saturated with nitrogen, and stored at –20 °C. When needed, the desired number of beads were taken with tweezers and put directly in the TX-TL mixture.
The average amount of POPC deposited on each bead was quantified by a modified version of the Stewart assay (S1). The Stewart reagent was prepared by mixing $FeCl_3 \cdot 6H_2O$ (1.36 g, 5 mmoles), KSCN (1.94 g, 20 mmoles), and NH_4Cl (1.07 g, 20 mmoles) in distilled water (50 mL). This mixture will contain KCl, which was absent in the original recipe. Five to ten beads were then put in chloroform (0.6 mL), to solubilize the POPC coating, and 0.2 mL of the Stewart reagent was poured on the top of the chloroform solution. After vortexing to mix the two phases, the samples were kept

undisturbed for about 30 minutes and the absorbance (470 nm) of the organic phase was read by a spectrophotometer. For calibration, different concentrations of POPC in chloroform solution were used (from 0 to 100 μM), giving an apparent molar extinction coefficient (ε_{470}) for the POPC/ammonium ferrothiocyanate complex of 0.0075 ± 0.0005 μM^{-1} cm^{-1} (compare with 0.0062 μM^{-1} cm^{-1} for DPPC, as reported in (12)). Beads covered by 5-15 nmoles POPC/bead were obtained (corresponding to about 70% of the expected amount, calculated on the basis of total added POPC and beads number). In a typical experiment, 5 POPC-coated glass beads were used to form liposomes in the TX-TL mixture (50-100 μL), so that the final POPC concentration was generally in the 0.25-1.5 mM range.

Liposome extrusion. When required, liposomes prepared by the thin film hydration method (film on glass beads), where extruded (1-2 passages) through one polycarbonate membrane (pore size: 800 nm; Nuclepore, Whatman) mounted on an autoclaved hand extruder (Liposofast, Avestin). The size of extruded liposomes was confirmed to be 800 nm (± 10%) by direct observation (confocal microscopy).

Preparation and use of freeze-dried liposomes. 10 mM POPC liposomes in water, prepared by the ethanol injection method using a 100 mM POPC in ethanol stock solution, were extruded through two polycarbonate membranes, with 400 nm pore size (Nuclepore, Whatman). The resulting vesicles were diluted 1:1 with water and freeze-dried in forms of 100 μL aliquots. Liposomes were then obtaining by re-hydrating the cake with 100 or 150 μL of TX-TL kit, to a final POPC concentration of 3.5-5 mM.

Liposomes prepared by the ethanol injection method. An ethanol solution of lipids (100 mM) was injected, by means of a Hamilton microsyringe, in the TX-TL mixture, in a 1:30 to 1:60 volume ratio, so that the final lipid concentration was 1.7-3.3 mM and the final ethanol amount was 1.7-3.3% v/v. The lipids used were: (*i*) POPC; (*ii*) POPC/sodium oleate 4/1 mol/mol. In the latter case, the stock solution was prepared from POPC/ethanol and sodium oleate/methanol stock solutions.
It should be noted that the original report on the "ethanol injection method" describes the preparation of small unilamellar vesicles by injecting diluted (< 25 mM) lecithin ethanol solution in an aqueous buffer (the final aqueous lecithin concentration is therefore very low, generally about or below 1 mM). This produces very diluted vesicles. As we have published recently, the average vesicle size and the size distribution of vesicles prepared by the ethanol injection method depend mainly from the concentration of POPC in the ethanol stock solution used to prepare the vesicles (S2). In particular, vesicle size increases when POPC concentration increases. Moreover, vesicle clusters can be also formed (S3) when high POPC concentration are employed (around 100 mM stock POPC concentration).

Glassware preparation for microscopy analysis.
All glassware was cleaned with ethanol and lint-free paper (Kimwipes Lite, Kimberly-Clark) to remove dust and contaminants from the surface. The thickness of an aqueous solution poured between the microscopy glass slide (25.4 × 76.2 mm) and the cover slip (24 × 24 mm) was measured by running *z*-scan profiles of a 1 μM calcein solution in PBS buffer, resulting to be: 5 μL/~8 μm; 10 μL/15 μm; 20 μL/30 μm. Liposome samples (10-20 μL) were analyzed accordingly.

Nile Red staining. When required, liposomes were stained by the hydrophobic dye Nile Red (ex. = 548 nm, em. = 620 nm in ethanol), which was directly added (1:5 v/v) to the liposomes, starting from a saturated solution in distilled water (approximate concentration: 1.7 μg/mL, as estimated from its K_{ow} (S4).

Confocal microscopy. eGFP Fluorescence measurements were carried out with a (*i*) Zeiss LSM 510 inverted microscope (dichroic HFT 488/NFT 515, emission filter LP 505; objective 63×); (*ii*) Leica TCS SP5 inverted microscope (dichroic RSP 500, emission window 500-600 nm; objective 63×). The eGFP emission spectrum was recorded with the Leica TCS SP5 instrument, by exciting the eGFP by the 488 nm line of an argon laser, and recording a series of fluorescence image for each of the narrow (5 nm) detection windows, from 500 to 600 nm. In the case of liposome membrane staining by Nile Red, the fluorescence signals from eGFP and Nile Red were recorded independently, by adopting a sequential scan mode (1^{st} scan – ex: 488 nm, int.: 50%, em: 500-530 nm; 2^{nd} scan – ex: 543 nm, int.: 90%, em: 600-700 nm; dichroic DD 488/543).
Each sample was analyzed by recording the fluorescence z-profile, the λ-profile (emission spectrum) and images of individual fluorescent vesicles were then taken. Although several vesicles were free floating in the solution (their images were difficult to capture), other vesicles spontaneously adhere to the glass-water interface and were easy to visualize. High-speed, low resolution, rectangular framed, bi-directional scanning was often employed.

Image acquisition and analysis. Digital images were acquired at 8-bits or 12-bits color depth, at 512 × 512 or 1024 × 1024 resolution, using monochromatic or color-coded LUTs. The fluorescence intensity was quantified directly by the acquisition/analysis Leica or Zeiss software, or by Image J (http://rsbweb.nih.gov/ij/). Images in full depth are shown in Figure S4*a-b*, whereas Figure S4*c* represents the same image as in panel *b*, but after brightness correction – to better visualize the liposomes (figures in the main text were treated similarly). Figure S5 show the fluorescence profiles of background and of the particle shown in Figure S4, and the corresponding comparison.

B. Estimation of co-entrapment probability

By following the treatment done in (S5), let us consider the expected number $N_{0,k}$ of the k species (k = 83, we consider here only macromolecules) entrapped inside a liposome with a volume V. This can be readily calculated from the bulk concentration of each specie $C_{0,k}$ and the Avogadro number (N_A), as $N_{0,k} = N_A V C_{0,k}$.
$N_{0,k}$ represents the average and the variance of the Poisson distribution describing the entrapment statistics.
We interrogate on what is the probability that the molecules of the k-th species are entrapped inside liposomes when $N_k > N_{0,k} + q\sigma$, where q is an integer (1, 2, or 3) and σ is the best estimate of the stochastic fluctuations around the mean $N_{0,k}$. For the Poisson distribution, $\sigma^2 = N_{0,k}$.

Preliminary analysis shows that the Poisson probability $p(N_k$, for $N_k > N_{0,k} + q\sigma$, q = 1, 2, or 3), for 1 μM liposome diameter differs from the analogous Gaussian probability of an amount minor than 5%. This difference decreases (to less than 2%) as the liposome diameter increases, and when q = 2 or 3.
Consequently, we used the Gaussian probability curve *for readily estimating* the probability of co-entrapment of k species at a local concentration that is 1, 2 or 3 times higher than the amplitude of the stochastic fluctuations around the expected concentration, because of the well-known properties of normalized Gaussian probability curve. In particular, the cumulative probability of events above 1, 2 or 3 standard deviations from the mean result to be, respectively, 15.9, 2.3 and 0.1% in the Gaussian curve.
The overall probability $\wp = \Pi_k p(N_k)$ of co-entrapping k species in the same liposome is readily calculated as product of the probabilities of entrapping each single specie, under the hypothesis of independent events. In the Table S1 the overall probability values for q = 1, 2, 3; and k = 83, 40, 20 are reported.

Table S1. Overall co-entrapment probabilities for k species, to give local intraliposome concentration above a threshold.

		Overall probability $\wp$		
q	$p\,(x > x_0 + q\sigma)$	$k = 83$	$k = 40$	$k = 20$
1	0.159	4×10^{-67}	1×10^{-32}	1×10^{-16}
2	0.023	4×10^{-137}	2×10^{-66}	1×10^{-33}
3	0.001	7×10^{-239}	2×10^{-115}	4×10^{-58}

Typical experimental conditions are: 1 mM POPC (head group area: 0.72 nm^2), 100 μL reaction volume, 1.5 μm liposome diameter. In these conditions we can estimate the presence of 3×10^9 unilamellar liposomes/sample, so that essentially no "super-filled" liposomes should be in the sample according to Poisson/Gaussian probability of co-entrapment. As it has been shown in (S6), however, if the solutes follow an occupancy distribution shaped by a power-law, the probability values increase significantly and the occurrence of "super-filled" liposomes is not more elusive.

C. Supplementary results

C1. Exploring the effect of dilution on TX-TL kits. We have firstly explored the protein synthesis efficiency of TX-TL mixtures (cellular extracts or PURE system) when they are diluted at different extent, to find the conditions where – in a given period of time, typically 2-4 hours – no eGFP can be detected. In particular, this was possible by a quantitative comparison between the fluorescence of the diluted TX-TL kit (in the presence of a plasmid encoding for the eGFP) and the fluorescence of a similar sample prepared without DNA (negative control). Experimental results indicate that the yield of eGFP rapidly decreases when the TX-TL kits are diluted, and becomes not detectable (i. e., statistically indistinguishable from a TX-TL mixture deprived of DNA) when their concentration is around 65% of the standard value (Fig. S1). Being the TX-TL reaction a multi-step, intermingled, association-dependent reaction, we ascribe this dramatic effect to the loss of complex formation efficiency between the several fundamental molecular components of the kit, due to their reduced concentration.

C2. Experimental conditions and descriptive statistics. The main results presented in this work derive from POPC liposomes prepared by the ethanol injection method and by the thin film hydration methods (on 2 mm glass beads). The experimental conditions are summarized in Table S2. These are based on pooling data that can be compared directly (222 vesicles). The total number of vesicles studies in this study is instead 324, and includes vesicles prepared by the extrusion techniques and observations after more than 4 hours. Typically, we found about 10 fluorescent vesicles/experiment.

Table S2. Statistical analysis of vesicles prepared by the ethanol injection method and thin film hydration method observed after 2-4 hours (222 vesicles).

#	Method	Lipid	[Lipid], mM	TX-TL kit	Dilution	Incubation time, hours	Mean diameter, μm*	Mean F_{in}/F_{out} (R)*
1	Ethanol injection	POPC	1.7-3.3	cell-extracts	0.6-1.0	2-4	1.8 ± 0.3	5.2 ± 1.9
2	Ethanol injection	POPC	1.7-3.3	PURE system	0.6-1.0	2.4	1.0 ± 0.3	7.4 ± 2.2
3	Thin film hydration (beads)	POPC	0.25-1.5	cell-extracts	0.6-1.0	2-4	1.7 ± 0.9	9.2 ± 7.2
4	Thin film hydration (beads)	POPC	0.25-1.5	PURE system	0.6-1.0	2-4	1.3 ± 0.5	9.7 ± 3.3

* mean ± standard deviation (n = 10, 38, 73, 101 for lines 1-4, respectively)

Vesicle size and internal fluorescence are quite dispersed, indicating that the vesicle formation and solute encapsulation are heterogeneous processes. Experiments carried out with differently diluted TX-TL kits can be easily compared by taking the ratio R between internal and external fluorescence ($R = F_{in}/F_{out}$), as a rapid estimate of the local intravesicle enhanced concentration. (As explained in the main text, it should be remarked that when the signal from a negative control sample is taken into account to correct the F_{out} value, the ratio R often diverges – "on/off" behavior. Nevertheless, here we prefer to use uncorrected R values as an easily achieved continuous variable and carry out statistical analysis for the whole dataset).
Statistical analysis (significance: $p < 0.05$) on the full dataset revealed that vesicle diameters depend both from the preparation method (ethanol injection or thin film hydration) and from the kind of TX-TL kit (cell extract or PURE system). The R values instead depend only from the preparation method (Figure S2a). Other experimental parameters like lipid concentration, TX-TL kit dilution, and incubation time have no statistical relevance for determining R and the vesicle size. The vesicle size distribution and the R distribution show peaks at ca. 1.25 μm and 7, respectively (Figure 2bc). Very high R values have been occasionally recorded, up to about 45. The R versus size dot-plot clearly shows that small vesicles reach high R values when compared with larger one, irrespective from the kit dilution (Figure 2d). This further suggests that the entrapment of solutes, and possibly the protein synthesis efficiency, might depend on surface-related phenomena.

In addition to the experiment carried out with POPC liposomes prepared by the injection method and on-beads thin film hydration method with short incubation time (2-4 hours), samples derived from longer incubation time (24 hours) were also explored, revealing no statistical difference with the short-time incubated samples. Moreover, similar TX-TL entrapment experiments were carried out in the following conditions:

- POPC liposomes prepared by the extrusion technique (800 nm)
- POPC liposomes prepared by the hydration of freeze-dried liposomes
- POPC/oleate (4/1 mol/mol) prepared by the ethanol injection method
- POPC liposomes stained with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine-B-sulfonate), ammonium salt (DOPE-Rh) (0.01 mol%) prepared by the on-beads thin film hydration method

The between-group statistical analysis of the ratio R showed a significant ($p < 0.05$) difference between the samples prepared by hydrating freeze-dried liposomes and all other methods (including injection and thin film hydration methods described above). This was somehow expected due to the well-know high capture efficiency of freeze-dried liposomes (S7). In this study we did not observe specific features concerning POPC/oleate vesicles.

Preliminary experiments on anchored liposomes entrapping the PURE system (prepared according to (S8), with minor modifications) confirm the observations done with free floating liposomes.

C3. Spectral analysis. Emission spectra of eGFP in bulk were obtained after its synthesis in the absence of liposomes, by using a Jasco FP-6200 spectrofluorimeter (λ_{ex}: 483 nm; λ_{em}: from 500 to 600 nm; emission and excitation slits: 5 nm) and by recording a $xy\lambda$-scan with a Leica TCS SP5 confocal microscope, as described above. Emission spectra of intravesicle eGFP were obtained also by microspectrofluorimetry. Preliminary analysis on normalized spectra allowed to pool together vesicles with similar eGFP spectral shapes. Characteristic emission spectra were then obtained by spectra averaging, in order to reduce the noise.
Emission spectra were normalized to one and deconvoluted as a sum of two Gaussian bands (see Equation S1) by means of the freeware software *fityk* (version 0.8.9) for Linux. The obtained

parameters are reported in Table S3. The deconvolution of intravesicle emission spectra were carried out by fixing the position of the peak to 511 and 531 nm, as obtained in the case of bulk eGFP (these data matches with the values reported in the literature, namely 512 and 530 nm (S9), whereas their width and amplitude were kept as variable parameters. Table S3 also reports some typical values obtained for intravesicle eGFP.

(Eq. S1)

$$F = F_0 + A_1 \exp\left[-\frac{(\lambda - \lambda_{max,1})^2}{2w_1^2}\right] + A_2 \exp\left[-\frac{(\lambda - \lambda_{max,2})^2}{2w_2^2}\right]$$

Table S3. Double gaussian fitting of the eGFP emission bands

	Peak 1			Peak 2			
	$\lambda_{max,1}$	w_1	A_1	$\lambda_{max,2}$	w_2	A_2	A_1/A_2
EGFP bulk (fluorimeter)	508.5 ± 0.1	10.7 ± 0.1	0.75 ± 0.05	530.1 ± 0.1	21.4 ± 0.1	0.35 ± 0.05	2.0
EGFP bulk* (microscope)	510.7 ± 0.5	7.9 ± 0.4	0.75 ± 0.14	530.8 ± 3.6	20.3 ± 3.1	0.41 ± 0.05	1.8
EGFP inside vesicles** (microscope)	511	9.8 ± 0.8	0.70 ± 0.04	531	18.1 ± 1.8	0.51 ± 0.04	1.4
EGFP inside vesicles** (microscope)	511	11.2 ± 1.1	0.51 ± 0.04	531	22.7 ± 1.6	0.57 ± 0.03	0.9
EGFP inside vesicles** (microscope)	511	6.3 ± 1.9	0.38 ± 0.11	531	16.8 ± 2.6	0.66 ± 0.07	0.6

* Best fit of the average spectrum, derived from pooling together 5 independent measurements
** Best fit of the average spectrum, derived from pooling together 2-6 vesicles displaying similar spectral shapes

C4. Further details about the modification of eGFP emission spectrum.
In addition to the explanation given in the main text, the cited report (S9) explain that the two Gaussian bands that constitute the eGFP emission spectrum derive from radiative transition from the excited electronic state to two vibrational levels of the fundamental electronic state. The modification of the spectrum in highly concentrated samples has been tentatively explained as due to a large overlap of the absorption and emission spectra or to structural perturbations in the cluster.

D. Possible explanation of similar observations
We cautiously suggest that the mechanism of solute accumulation inside liposomes described in this work might be extended to a previous published intriguing observation (S10), where it has been reported that giant vesicles entrapping components from an undiluted TX-TL kit displayed enhanced fluorescence (ca. 2×) when compared with the fluorescence of the bulk, especially in the first 3 hours.

E. Supplementary figures

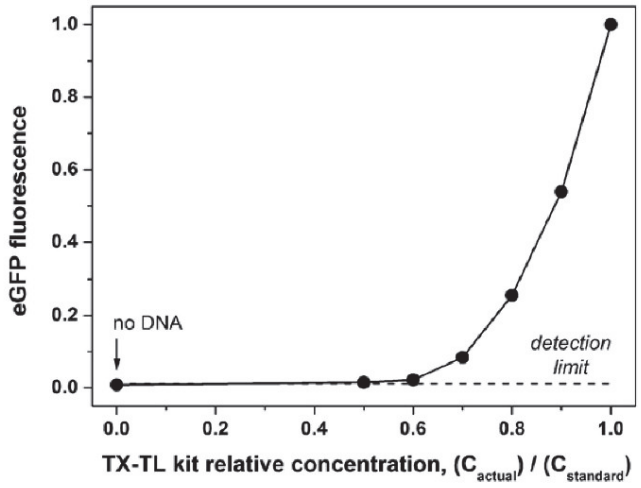

Figure S1. Typical variation of eGFP synthesis with the dilution of the TX-TL kit (PURE system or cell extracts gives slightly different profiles). The x-axis reports the relative TX-TL kit concentration (actual concentration/standard concentration), i.e., the TX-TL kit concentration increases moving from left to right (e.g., $x = 0.5$ means that the TX-TL kit has been diluted 1:1 with buffer; $x = 0.8$ means that 4 volumes of the TX-TL kit has been mixed with 1 volume of buffer, and so on). The $x = 0$ point represents instead the fluorescence of the sample prepared in the absence of DNA. The production of eGFP rapidly decreases as soon as the kit is diluted (moving from right to left), and become undetectable by confocal microscopy when the TX-TL kit is diluted 0.5-0.6×. In our conditions, such mixture fluorescence values were indistinguishable from the negative control sample (no DNA).

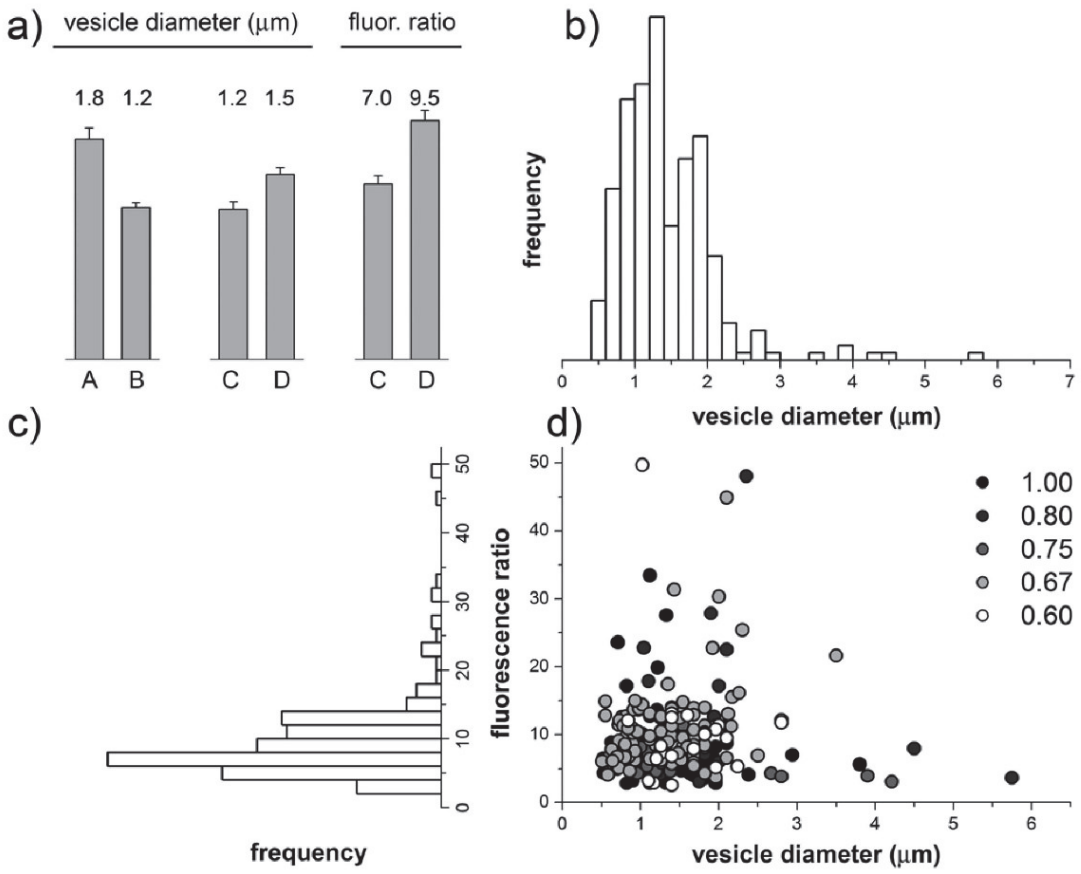

Figure S2. Statistical analysis of vesicles prepared by the ethanol injection method and thin film hydration method, observed for short and long incubation time (> 4h). Note that Table S2 refers to short incubation time (2-4h) only. (a) Statistical analysis reveals which of the five experimental parameters here analyzed (liposome preparation method, nature of the TX-TL kit, dilution of the TX-TL kit, liposome concentration, incubation time) affect the experimental outcomes in terms of liposome diameter and on the F_{in}/F_{out} ratio (R) of the fluorescent (eGFP producing) liposomes. Liposome diameter resulted to be dependent ($p < 0.05$) from the nature of the TX-TL kits (A: cell extract; B: PURE system) and from the nature of the TX-TL kit used (C: injection; D: film hydration). The ratio R depends only from the preparation method. The other experimental parameters do not affect the diameter and the R value. (b-c) Pooled liposome diameter and R values distributions. (d) Relation between the R values and the liposome diameter of eGFP producing vesicles, grouped according to the TX-TL kit dilution (from 0.6× to 1×). Note that smaller liposomes tend to display higher R values.

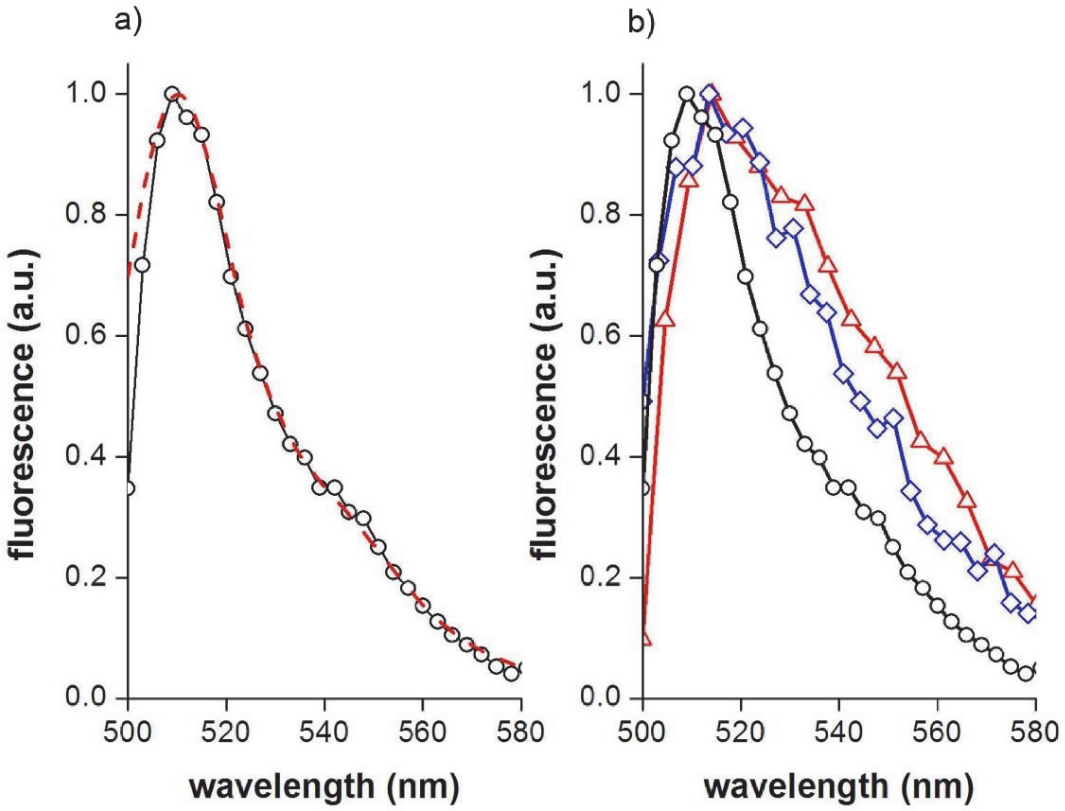

Figure S3. (a) Comparison between "bulk" eGFP emission spectra obtained by classical cuvette fluorimetry (red dotted line) and microspectrofluorimetry (black empty circles). (b) Comparison between the "bulk" eGFP emission spectrum, obtained by microspectrofluorimetry (black empty circles), and the intra-liposome eGFP emission spectrum, in two typical cases (blue diamonds, red triangles). In contrast to "bulk" spectra, intra-liposome spectra have small signal-to-noise ratio due to the low number of pixels that represent the liposome. Here averages of 2-5 similar spectra are presented.

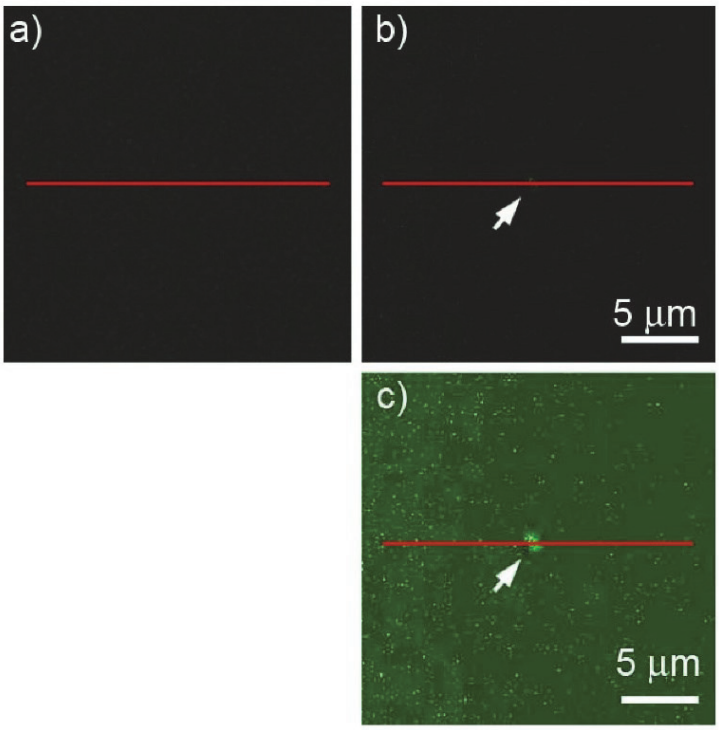

Figure S4. Raw (a,b) and highly contrasted (c) confocal microscopy images: (a) negative control sample (without DNA); (b,c) cell extracts TX-TL kit (0.65×) in the presence of liposomes prepared by the ethanol injection method. Panel (c) is the same as (b), after digital manipulation. Intensity profiles along the red lines are presented in Figure S5.

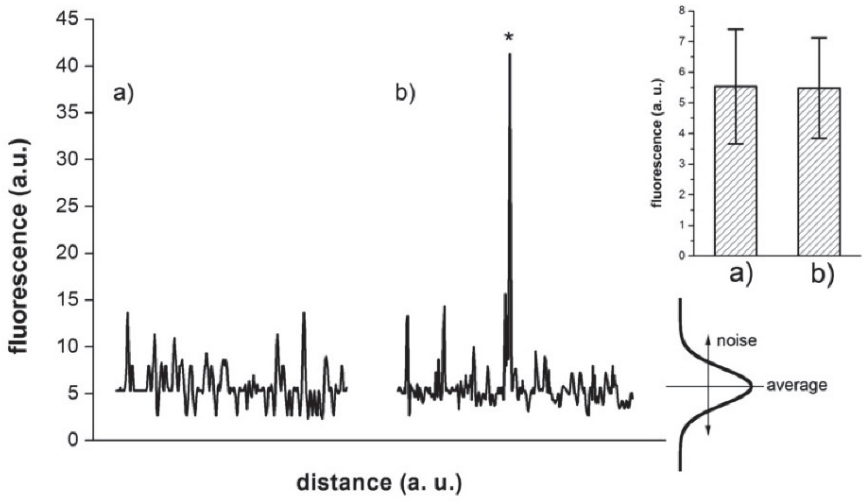

Figure S5. Fluorescence intensity profiles along the red lines in Figure S4, panels (a,b). The average values of fluorescence background (shown as the two bars on the right) are statistically indistinguishable.

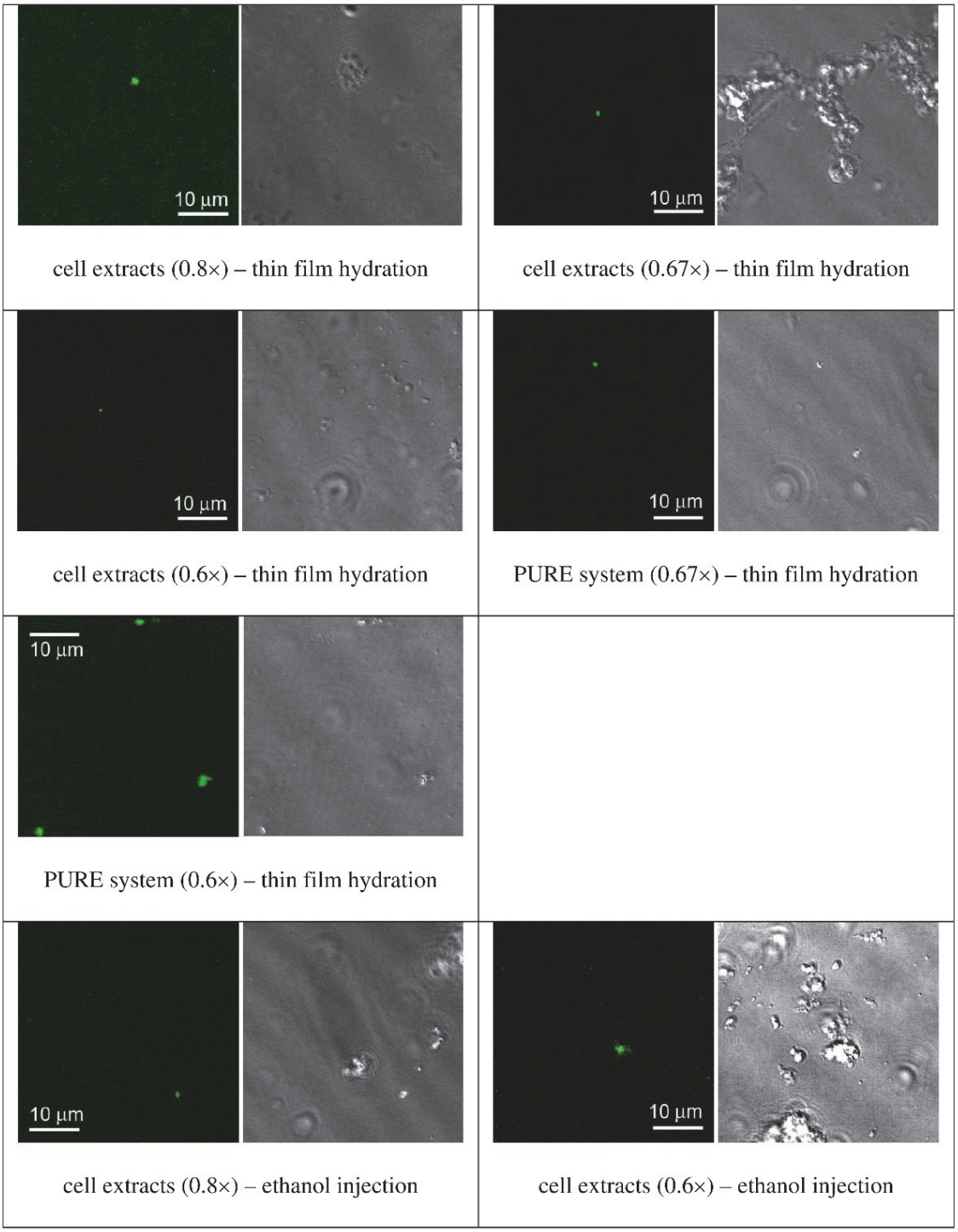

Figure S6(a). Confocal images of eGFP producing liposomes prepared in different conditions.

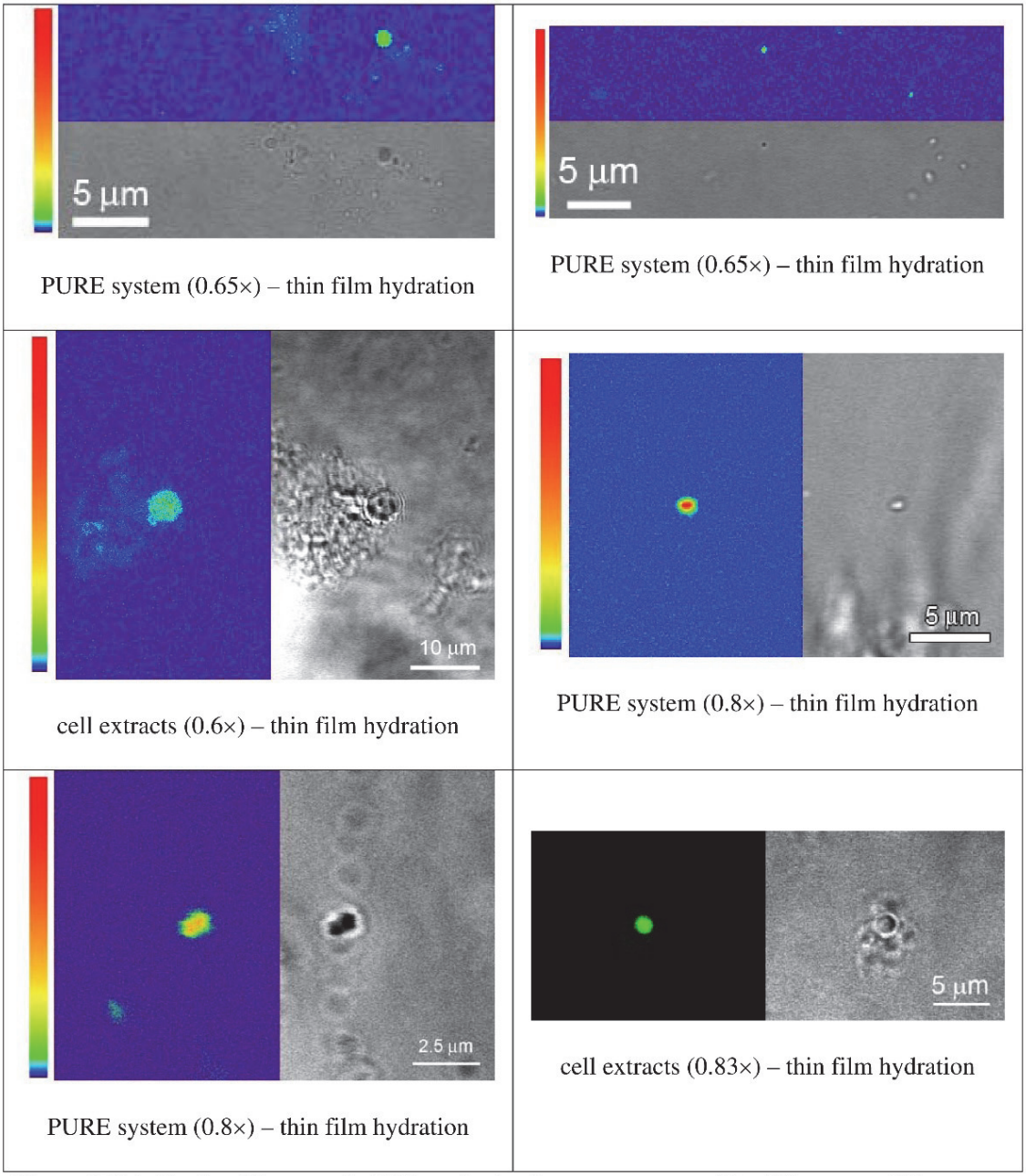

Figure S6(b). Confocal images of eGFP producing liposomes prepared in different conditions.

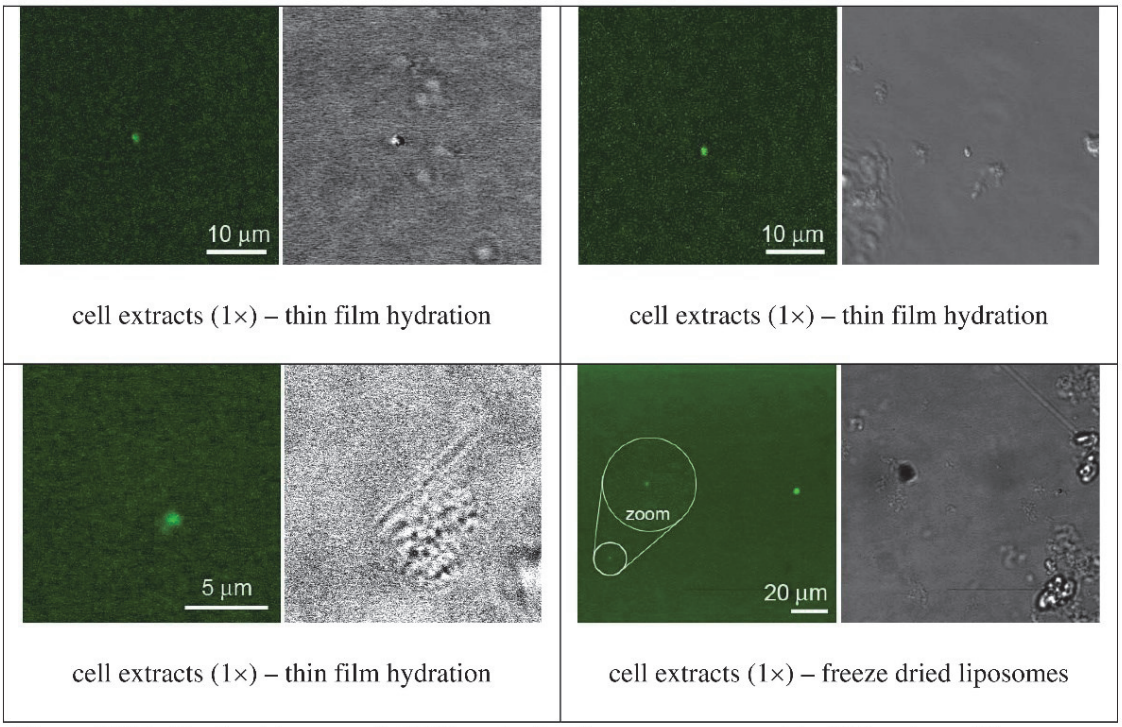

Figure S6(c). Confocal images of eGFP producing liposomes prepared in different conditions.

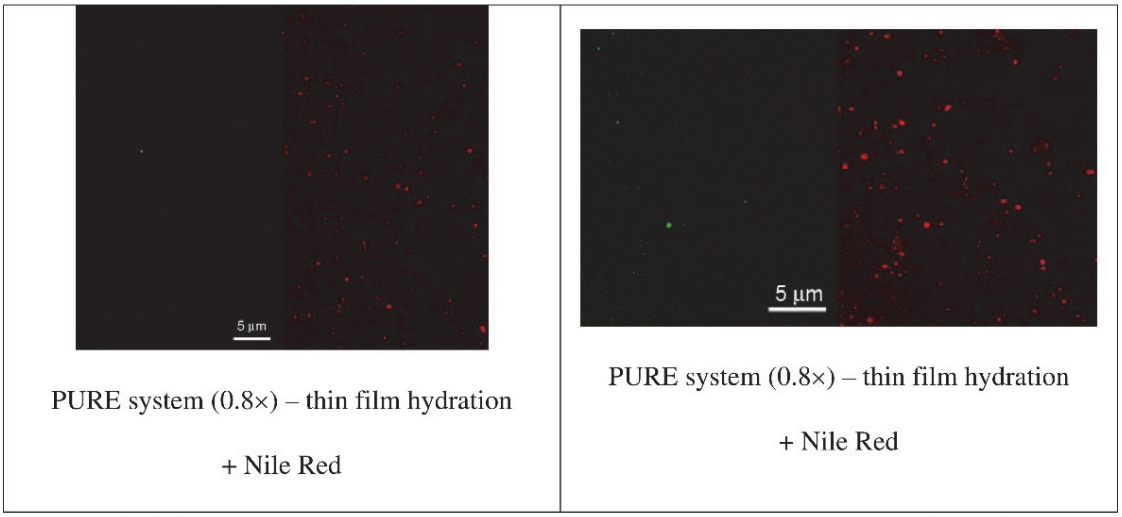

Figure S6(d). Nile Red staining (right panels) of eGFP producing liposomes (left panel) reveals that the large majority of liposomes do not synthesize the protein.

F. Supplementary References

S1. Stewart, J.C.M. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. *Analytical Biochemistry* **104**, 10–14 (1980).

S2. Domazou, A. S. & Luisi, P. L. Size distribution of spontaneously formed liposomes by the alcohol injection method. *J. Lipos. Res.* 12, 205-220 (2002).

S3. Stano, P., Bufali, S., Domazou, A. S. & Luisi, P. L. Effect of tryptophan oligopeptides on the size distribution of POPC liposomes: a dynamic light scattering and turbidimetric study. *J. Lipos. Res.* 15, 29-47 (2005).

S4. http://www.chemspider.com/58681

S5. Souza, T., Stano, P. & Luisi, P. L. The minimal size of liposome-based model cells brings about a remarkably enhanced entrapment and protein synthesis. *Chembiochem* **10**, 1056-1063 (2009).

S6. Luisi, P. L. *et al.* Spontaneous protein crowding in liposomes: a new vista for the origin of cellular metabolism. *Chembiochem* **11**, 1989–1992 (2010).

S7. Shew, R. L. & Deamer, D. W. A novel method for encapsulation of macromolecules in liposomes. *Biochim Biophys Acta.* **816**, 1–8 (1985).

S8. Lohse, B., Bolinger, P. Y. & Stamou, D. Encapsulation efficiency measured on single small unilamellar vesicles. *J. Am. Chem. Soc.* **130**, 14372–14373 (2008).

S9. Cinelli, R. A. *et al.* The enhanced green fluorescent protein as a tool for the analysis of protein dynamics and localization: local fluorescence study at the single-molecule level. *Photochem Photobiol.* **71**, 771–776 (2000).

S10. Nomura, S. M. *et al.* Gene Expression within Cell-Sized Lipid Vesicles. *Chembiochem* **4**, 1172–1175 (2003).

3.2 Pubblication 3

Encapsulation of Ferritin, Ribosomes, and Ribo-Peptidic Complexes Inside Liposomes: Insights Into the Origin of Metabolism.

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CONFERENCE REPORT

Encapsulation of Ferritin, Ribosomes, and Ribo-Peptidic Complexes Inside Liposomes: Insights Into the Origin of Metabolism

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Abstract Here we summarize the main results of our latest investigation on the spontaneous encapsulation of proteins (ferritin) and ribosomes inside lipid vesicles. We show that when vesicles form in a solution containing some macromolecules (even at low concentration), in contrast to the expectations, a few but measurable number of vesicles is able to capture a very high number of solutes, up to 60 times the external concentration. We also show preliminary evidences on the encapsulation of additional solutes (ribo-peptidic complexes, fluorescent proteins and enzymes), and shortly present our current approach aimed at exploiting this phenomenon. In particular, we would like to reveal how the formation of compartments can trigger effective intra-vesicle reactions starting from diluted solutions. Although the mechanistic details for this phenomenon are still missing, we claim that these new evidences are highly relevant for the origin of the first functional cells in primitive times.

Keywords Liposomes · Encapsulation · Ferritin · Ribosomes · Power-law · Metabolism

The Formation of Primitive Cells

One of the key and still unanswered questions in origin of life research is the formation of primitive cells. There is indeed a gap between studies of the early chemical evolution and the

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biological pathways starting from the last universal common ancestor. In particular, since it is well accepted that semi-permeable membrane vesicles represent the most plausible precursors of cells (Deamer and Dworkin 2005), one open question would be the time at which these compartments came into the picture as hosts for the first forms of metabolism.

For the "metabolism first" and "replication first" scenarios, we are faced with a problem: how could all the molecules - which were firstly developed in solution - become simultaneously incorporated, later on, into a closed compartmentalised structure?

Alternatively, in order to let the first forms of metabolism arise inside closed compartments, and become more complex later, it is necessary that primitive membranes have an efficient, directional, and preferably controlled material exchange with the environment. But simple vesicular structures lack control of membrane permeability. So, either way, we are facing a conceptual conundrum.

Starting from our previous considerations on the encapsulation of solutes inside lipid vesicles (Luisi 2006), we recently started a direct investigation of this process, aimed at understanding the role of physical self-organization in the onset of cell-like particles. We carried out a systematic investigation based on cryo-transmission electron microscopy (cryo-TEM) of solute-containing vesicles (Luisi et al. 2010; Souza et al. 2011), and we are currently expanding these studies to include other solutes that cannot be analyzed by cryo-TEM. With surprise, we discovered an unexpected phenomenon that might help to clarify some aspects of the above-mentioned conundrum.

In this contribution, we summarize our recent studies on the spontaneous assembly of primitive cell-like structures, by using lipid vesicles (liposomes) as cellular models. We show that lipid vesicles can spontaneously capture a very high number of macromolecular solutes even when formed in diluted solutions, overcoming one of the major problems in prebiotic chemistry, i.e. the expected low concentration of solutes. As a result of this intriguing behavior, these solute-rich vesicles could be able to facilitate and support the emergence of a cellular metabolism (Luisi 2012), thanks to the molecular richness in their aqueous core. We believe that our investigation reveals an important new concept for the origin of cells and more in general for self-organizing systems.

The Encapsulation of Ferritin and Ribosomes Inside Liposomes

Our experimental model consists in the formation of lipid vesicles in a solution of a macromolecular solute. Vesicles, formed by 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) or POPC/cholesterol mixtures, were formed by two classical methods, namely thin film hydration (with or without extrusion to a definite size) and ethanol injection. The solutes present in the solution can be entrapped within the lipid vesicles at the moment of their formation. Due to the impermeability of lipid membranes to such large molecules, the analysis of the liposome contents provides realistic insights in the physics of solute encapsulation. In order to detect the molecular content of each vesicle, at the level of a single molecule, it is necessary to use solutes that can be individually visualized by cryo-TEM, which is the best method for imaging lipid vesicles. Based on our previous studies of the 'matrix effect' (Berclaz et al. 2001a,b), we first used ferritin (Luisi et al. 2010), a 12.5 nm large water-soluble protein which consists of 24 subunits enclosing a spherical cavity where iron is stored in form of hydrous ferric oxide phosphate (ca. 4,000 iron atoms/ferritin). Thanks to its iron core, ferritin molecules can be easily detected by cryo-TEM and counted individually. Next, we also used ribosomes (Souza et al. 2011), that can be visualized by cryo-TEM even if they miss heavy metal atoms. The choice of entrapping ribosomes was

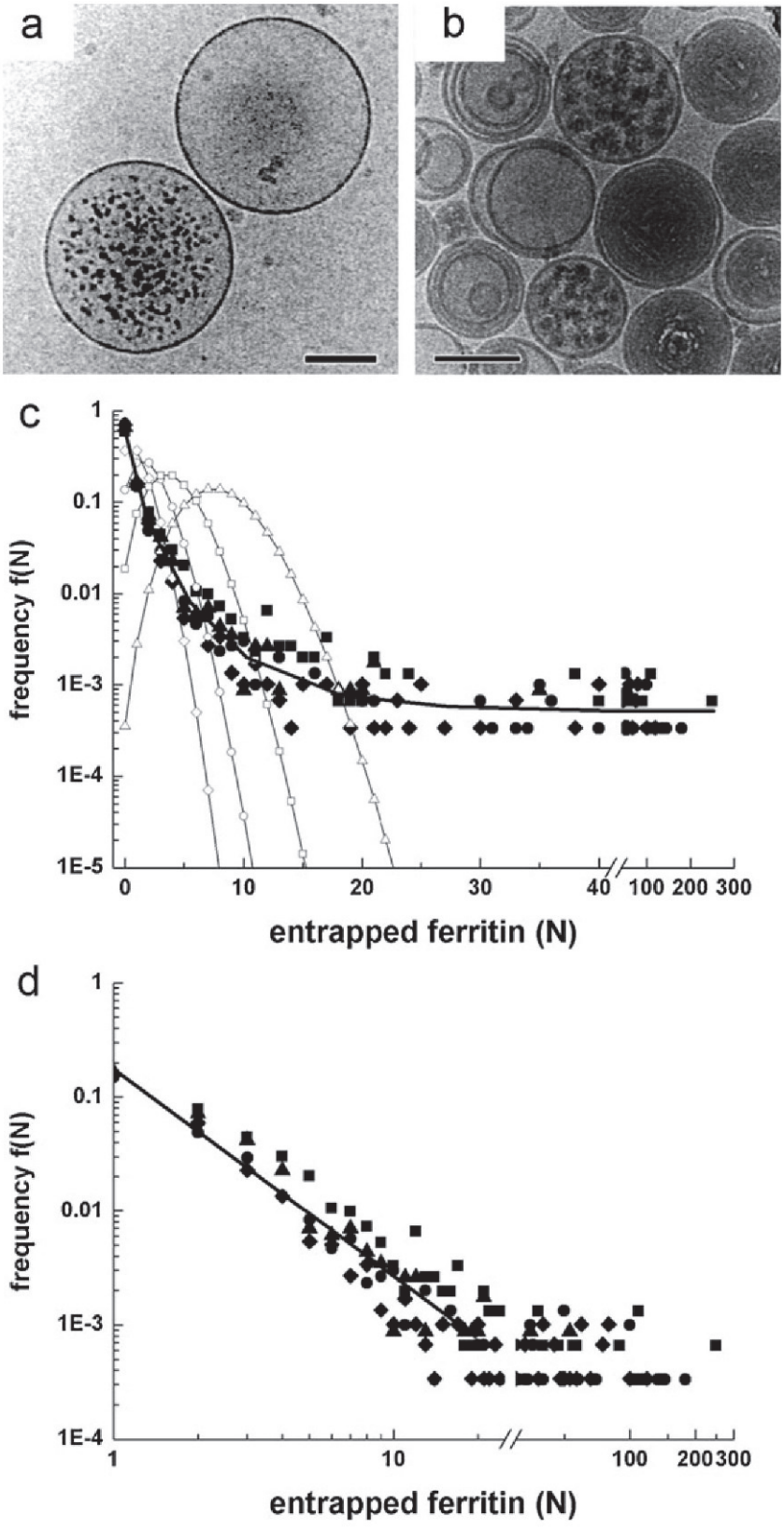
because of their relevance as key compounds for protein synthesis and therefore for the development of primitive functional cells.

The starting point of our discussion is the formulation of the simplest hypothesis for solute entrapment inside vesicles. When a vesicle of volume V is formed in a solution containing a certain solute with concentration C_0, this process can be considered as equivalent to sampling the solution. It is then expected that, on average, a vesicle will contain N_0 molecules, where $N_0=N_A\ V\ C_0$ (N_A being the Avogadro number). Clearly, not all vesicles will contain N_0 molecules, because of stochastic fluctuation of the solute concentration. These variations around the average value N_0 are described by a Poisson distribution, which gives the probability of finding a vesicle (with volume V) containing N molecules instead of N_0. For example, consider a 100 nm (diameter) vesicle that forms in a 9.5 μM ferritin solution. In these condition, $N_0=3$. According to the Poisson distribution, it is expected that about 5 % of vesicles are empty, most of the vesicles will contain 3 ferritin molecules, and the probability of finding a vesicle with many more ferritin molecules (let us say, 10 or more) will be very low. A bell-shaped curve will describe the solute occupancy distribution.

These being the expectations let us see what we observed. As shown in Fig. 1a–b, both for ferritin and ribosomes, the direct observation of liposome samples after solute entrapment revealed an unexpected outcome. In particular, the first important observation was that the majority of vesicles (>80 %) were empty. The frequency of solute-filled vesicles decreases as the number N of entrapped molecules increases, and striking enough, we observed that about 0.1–1 % of vesicles contained a very high number of solutes (N>20, up to ca. 300), in clear contrast to the prediction of the Poisson law. As pictorially evident in Fig. 1a–b, the outcome of the spontaneous entrapment of these macromolecules inside lipid vesicles can be emphatically described as 'all-or-nothing' pattern, rather than by a bell-shaped solute occupancy distribution.

Figure 1c shows the experimental (versus Poisson) distribution of vesicles containing ferritin, obtained after directly counting the number of ferritin molecules inside 7,700 vesicles, prepared from different ferritin solution (C_0 from 4 to 32 μM). Similar results have been obtained in the case of ribosome containing vesicles (ca. 400 vesicles; C_0 ranging from 0.48 to 8 μM). It can be seen how the experimental distributions deviate substantially from the Poisson curves. In particular, the frequency of vesicles containing 0, 1, 2, … N solutes decreases monotonically, but very slowly. Thanks to this specific patter, it turns out that the existence of 'super-filled' vesicles is not as improbable as calculated by the Poisson curve. For example, the Poisson probability of finding a 100-nm vesicle containing 50 ferritin molecules, when C_0=8 μM, is about 10^{-46}, whereas we found that actually we can find one such vesicle out of 1,000 vesicles (10^{-3}). If the data of Fig. 1c are plotted in a bi-logarithmic plot, as shown in Fig. 1d, it becomes evident that the solute occupancy distribution seems to follow a power law profile. We are currently investigating the generative mechanism of this power law distribution, and its connection with the mechanism of vesicle formation and solute capture (Mavelli et al., manuscript in preparation).

Fig. 1 Entrapment of ferritin (**a**) and ribosomes (**b**) inside lipid vesicles, as shown by cryo-TEM imaging (*size bar*: 100 nm). Note that both ferritin and ribosomes, although present at high concentration inside the vesicles, are not aggregated. Panel (**c**): Ferritin occupancy distribution profiles, obtained for 4 different experiments, where ferritin concentration (C_0) was 4 (*diamonds*), 8 (*circles*), 16 (*squares*) or 32 μM (*triangles*). Filled symbols: experimental distribution; empty symbols connected by a line: expected Poisson distribution. Similar results have been obtained for ribosome entrapment. Note the bell-shaped Poisson curve, centered on the expected number of entrapped ferritin (N_0) in vesicles of diameter 100 nm, versus the monotonally decreasing experimental curve. Panel (**d**) shows the same experimental data presented in panel (**c**) but rendered in a bi-logarithmic plot. This makes evident that the ferritin occupancy distribution follows a power law. Panels (**a**,**c**,**d**) reproduced from Luisi et al. (2010) with permission from Springer. Panel (**b**) reproduced from Souza et al. (2011) with permission from Springer


a
b
c
frequency f(N)
1
0.1
0.01
1E-3
1E-4
1E-5
0
10
20
30
40
100
200
300
entrapped ferritin (N)
d
frequency f(N)
1
0.1
0.01
1E-3
1E-4
1
10
100
200
300
entrapped ferritin (N)

But there is a second and more important consequence of deviation from the Poisson law in favor of the power law. In fact, despite the fact that most of the vesicles are empty, the filled vesicles – actually 'super-filled' – contain a number N of solutes that exceed the expected value N_0. This means that the local (intra-vesicle) solute concentration C is higher than the bulk concentration C_0. In other words, these vesicles have actively recruited solutes during their formation, bringing about a concentration enhancement of factors up to about 60. The local concentration then might rise from a rather diluted value, e.g., 10 μM, to 0.6 mM. By considering the volume occupied by the solutes confined in the tiny intra-vesicle lumen, it has been estimated that in many cases – especially for the smaller vesicles – these values correspond to crowding concentration, typical of living cells.

A more detailed discussion of these data can be found in the original articles (Luisi et al. 2010; Souza et al. 2011), as well as a proposal for the mechanism underlying the effect of solute 'super-concentration', up to crowding conditions. Here it is useful to add a short comment on the fact that such anomalous behavior helps explain in a rather clear way another set of experimental data obtained by encapsulating the complex transcription/translation machinery (about 80 macromolecular compounds, plus amino acids, nucleotides, etc.) inside 200 nm vesicles (Souza et al. 2009). In order to explain the success of intra-vesicle protein production (that was against the theoretical prediction of simultaneous co-entrapment of 80 different macromolecules inside such small vesicles) we made the hypothesis that the local concentration of these molecules should have reached an enhancement factor of about 20. If solutes are encapsulated according to a power law rather than a Poisson law, this enhancement factor becomes realistic (for a stochastic simulation, see Lazzerini-Ospri et al. 2012) and would explain how 200 nm vesicles can indeed entrap a complex molecular 'soup' and concentrate these compounds inside. Cryo-TEM images of vesicles formed in this way were also published (Souza et al. 2011).

The Case of Ribo-Peptidic Complexes

Having shown the intriguing encapsulation pattern of ferritin and ribosomes, we reasoned that an additional proof of concept on the relevance of this phenomenon for the origin of life, would consist in the extension of these results to the encapsulation of 'primitive' compounds. In the past years, we investigated the formation of very simple ribo-peptidic complexes, which roughly resemble ribosomes (D'Aguanno 2009). These are ionic complexes formed between ribonucleic acids, like tRNA or rRNA, mixed with polycations, such as poly-L-lysine or poly-L-arginine. Since these complexes can be visualized by cryo-TEM, we checked whether they can be also entrapped inside lipid vesicles formed in situ as happens for ferritin and ribosomes.

Firstly, we optimized the method of complex preparation by varying the concentration and the molar ratio of the compounds used (20 nt RNA, and 7.5 kDa poly-L-arginine), in order to get a population of narrowly distributed particles, with a diameter of about 5 nm (Fig. 2a, see details in the figure caption). Then we used these particles, which mimic primitive ribo-peptide complexes, for an experiment of solute encapsulation. The observation of a few vesicles quite filled with the complexes, together with a large number of empty vesicles (Fig. 2b), agrees with previous results obtained with ferritin and ribosomes. This experiment, although missing a quantitative statistical analysis, reveals that a general encapsulation mechanism operates to produce the 'super-filled' vesicles, and that it is independent of evolved macromolecular sequences, being also observed for very simple molecular moieties.

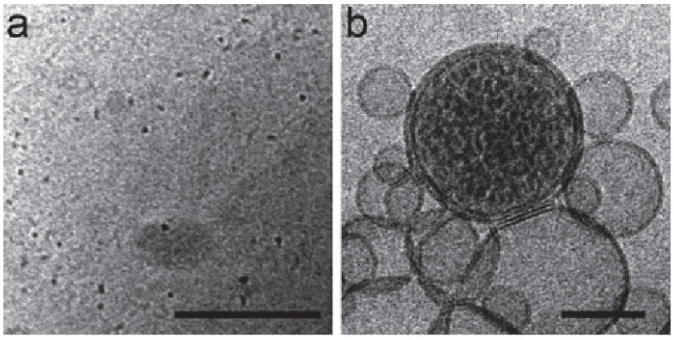


Fig. 2 Ribo-peptidic complexes (**a**) and their encapsulation inside lipid vesicles (**b**), pre-stained with phosphotungstic acid, as shown by cryo-TEM imaging, (*size bar*: 100 nm). Ribo-peptidic complexes were prepared by mixing 15 μM of 20-nt double stranded RNA (i.e., 40 residues) with 6.6 μM of 7.5 kDa poly-L-arginine (39 residues long) in 20 mM bicine (sodium salt, pH 8.5), corresponding to a molar fraction of positive charges equal to 0.3. The suspension of ribo-peptidic particles, shown in (**a**) was then incubated with 1 % phosphotungstic acid (after adjustement of the stock solution pH to 7.6), and later used to prepare POPC vesicles (**b**). Vesicles were prepared, manipulated, and imaged as reported in Luisi et al. (2010) and Souza et al. (2011)

Work in Progress

Until now our research has been focused on sub-micron vesicles and on solutes that can be visualized by cryoTEM, in order to demonstrate the deviation of the encapsulation process from the expected Poisson law and verify by direct imaging the existence of 'super-filled' vesicles. Currently we are working on expanding these initial studies to larger vesicles and fluorescent solutes. This will allow us to use standard fluorescence microscopy (confocal) to monitor not only static molecules like ferritin, but to follow dynamical processes as single- or multi-enzyme processes. The only requisite is that the solutes themselves or the product of the reaction they catalyse must be fluorescent. For this aim we are currently involved in investigating the encapsulation of proteins and other solutes in micrometer sized vesicles formed by film hydration (on glass beads) or ethanol injection.

Preliminary results indicate that a wide range of solutes actually behave as shown for ferritin, ribosomes and ribo-peptidic complexes: most vesicles turn out to be empty, and a few vesicles contain a number of solute molecules much higher than expected. Due to the larger vesicle size, the expected number N_0 of entrapped solute is now high (for vesicle diameter of 1 μm and C_0=9.5 μM, N_0~24,000). Nevertheless, preliminary data (see Fig. 3a-b), obtained for fluorescently labeled albumin and dextranes, phycoerythrin, allophycocyanin, and for the enzymes carbonic anhydrases and protease K, all show that it is possible to observe – although in a few vesicles - local concentration enhancement factors from 3 to 4 (most frequent, see Fig. 3c-d) to 10–15 (rarely) (D'Aguanno et al., work in progress). According to these first data, it appears that by increasing vesicle size, the magnitude of the 'super-concentration' effect is slightly reduced when compared with sub-micron vesicles, suggesting a physical role related to the surface-to-volume ratio. We have not yet investigated the case of giant vesicles (diameter above 5 μm), but an interesting report from the group of Keating (Dominak and Keating 2007) shows that similar (but weaker) effects still occur in giant vesicles.

Most interestingly, the possibility of fluorescence detection of super-filled vesicles triggered us to simulate primitive events of spontaneous intra-vesicle solute concentration by the protein synthesis model (specifically, green fluorescent protein synthesis). Vesicles are formed in situ in sluggishly reacting, diluted transcription/translation machinery – a

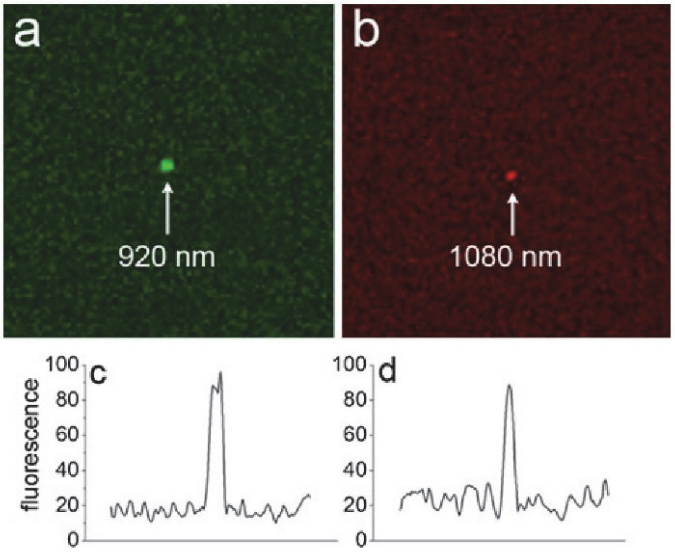


Fig. 3 Entrapment of fluorescent proteins and dextranes inside lipid vesicles. Fluorescence micrographs, obtained by confocal laser scanning microscopy, showing (**a**) 920 nm POPC vesicle containing fluorescein-labelled bovine serum albumine (BSA-FITC); and (**b**) 1,080 nm POPC vesicle containing phycoerythrin (PE). Shortly, POPC vesicles were prepared by hydrating POPC films deposited on 10 glass beads (2 mm diameter, 16 nmoles POPC/bead) with a solution (200 μL) of 1 μM BSA-FITC (or PE). After vesicle formation, the sample has been visualized without any additional treatment. Free solutes are present outside vesicles. Panels (**c**) and (**d**) show the quantitation of fluorescence along a line crossing the vesicle in panels (**a**) and (**b**), respectively. The fluorescence contribution of non-entrapped BSA-FITC (or PE) accounts for 18 (or 22) fluorescence a.u., whereas the internal BSA-FITC (or PE) accounts for about 96 (or 89) fluorescence a.u. In this case, the solutes were concentrated by a factor 5.3 (or 4.0). In other, rarer cases it was possible to observe concentration factors of about 10–15

model of an inefficient metabolic network. If vesicles were able to concentrate in their core all the molecules needed to synthesize protein, so that their internal concentrations become 10–20 times higher than in the bulk, an efficient protein synthesis could be observed only inside liposomes, whereas almost no reaction should occur in the outer environment (Stano et al., work in progress). This would demonstrate in a clear-cut way the active role of compartments for triggering an efficient metabolism starting from diluted solution, and emphasize the role of confinement and local concentration in the early steps toward the first living cells.

Concluding Remarks

In conclusion, we believe that we have brought new evidences on the question of the origin of functional cells in primitive times. We have observed, by cryo-TEM imaging, that when vesicles form spontaneously in a solution of diluted solutes (of different chemical nature and molecular weight), whereas most of the vesicles are empty, few of them are able to entrap a very high number of solutes, so that a very high internal concentration of that solute is reached (possibly in the crowding regime), against the entropic expectations. This shows that vesicles might actively favor the onset of a compartmentalized metabolism on the basis of purely physico-chemical forces. Although the mechanistic details are still unclear, experimental evidences clearly point to the involvement of surface effects and cooperative behavior based on weak solute/solute and solute/membrane interactions. Finally, it should be

recalled that the spontaneous formation of cell-like structures, based on the observation reported here, can pave the way to direct investigations in simulated diluted primitive conditions, so that the consequences on reaction efficiencies can be experimental examined.

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References

Berclaz N, Blöchliger E, Müller M, Luisi PL (2001a) Matrix effect of vesicle formation as investigated by cryotransmission electron microscopy. J Phys Chem B 105:1065–1071

Berclaz N, Müller M, Walde P, Luisi PL (2001b) Growth and transformation of vesicles studied by ferritin labeling and cryotransmission electron microscopy. J Phys Chem B 105:1056–1064

D'Aguanno E (2009) Studi su modelli prebiotici di ribosomi (Master Thesis). University of Roma Tre, Rome

Deamer DW, Dworkin JP (2005) Chemistry and physics of primitive membranes. Top Curr Chem 259:1–27

Dominak LM, Keating CD (2007) Polymer encapsulation within giant lipid vesicles. Langmuir 23:7148–7154

Lazzerini-Ospri L, Stano P, Luisi PL, Marangoni R (2012) Characterization of the emergent properties of a synthetic quasi-cellular system. BMC Bioinforma 13(Suppl 4):S9

Luisi PL (2006) The Emergence of Life: from Chemical Origin to Synthetic Biology. Cambridge University Press, Cambridge

Luisi PL (2012) On the emergence of metabolism. Helv Chim Acta, in press

Luisi PL, Allegretti M, Souza T, Steineger F, Fahr A, Stano P (2010) Spontaneous protein crowding in liposomes: A new vista for the origin of cellular metabolism. ChemBioChem 11:1989–1992

Souza T, Stano P, Luisi PL (2009) The minimal size of liposome-based model cells brings about a remarkably enhanced entrapment and protein synthesis. ChemBioChem 10:1056–1063

Souza T, Steiniger F, Stano P, Fahr A, Luisi PL (2011) Spontaneous crowding of ribosomes and proteins inside vesicles: A possible mechanism for the origin of cell metabolism. ChemBioChem 12:2325–2330

4 Discussion

4.1 Experimental approach to spontaneous self-organization of super-filled vesicles

The experimental approach presented in this dissertation is part of a broad research program on the study of the *Minimal Cell* (Luisi and Varela,1990; Oberholzer et al., 1995; Luisi et al.,2002), the minimal cell-like structure displaying at least some living properties as self-maintenance and self-reproduction. The program was originally devised for Origin of Life studies (Luisi et al., 2006), but it has been recently recognized as a leading program also in Synthetic Biology* (De Lorenzo and Danchin, 2008).

The *minimal cell* project included prebiotic, modern biochemical- and biophysical approaches. One of the most successful one is that one aimed at constructing "semi-synthetic minimal cells" (Luisi et al., 2006) by entrapping modern biological molecules (DNA, RNA, ribosomes, enzymes, …) inside vesicles, in order to trigger some chemical and physical mechanisms such as basic metabolism, or lipid synthesis (and then vesicle growth). In the past, despite the success of several revealing studies on the reconstitution of biochemical transformations inside lipid vesicles (reviewed by Stano et al., 2011) not much attention has been given on biophysical aspects concerning the origin (the assembly) of primitive cells. In particular, the spontaneous assembly of primitive cells is taken for granted, probably because of the well-known fact that vesicles form spontaneously in solution. However, a primitive cell is not only made of a lipid shell. Primitive cells must contain, in their aqueous core and probably also in their membrane, those components that assure (bio)chemical functionality to the whole assembly. The additional evidence that it is possible to encapsulate molecules inside vesicles, just by letting lipids assemble in the presence of such molecules, gives the perception that the formation of a complex, multi-component compartmentalized system is an easy task. Indeed, self-organization is a powerful route to primitive cells, but when one looks at the details, there are still some unanswered questions in the field.

The goal of this research project is therefore the understanding of the mechanisms at the base of the origin of the first primitive cellular metabolism. Specifically, the question that

* It has been already remarked, in the first chapter, that the aim of this ever-growing research field is constructing cellular models with the minimal and sufficient number of compounds to be defined alive, namely it is supposed to be able to auto-maintaining (metabolism), auto-reproducing and evolving. It is important to clear up that the coexistence of all the three features in synthetic systems is difficult to obtain, and one can conceive approximations, e.g., make protocells able to auto-maintaining but not to evolving. Having this kind of simple and not completely efficient systems is an advantage from the bioethical perspective, because we do not incur to the risk to have an incontrollable cell-multiplication.

drew the work was if the membrane closure – during spontaneous vesicle formation – could have played a role on the emergence of a protocell capable of some rudimentary metabolism.

Here it is useful to recall the two opposite scenarios one can imagine (see Figure 4.1): (1) elementary compounds are encapsulated inside vesicles and later develop more complex molecules, and eventually a sort of metabolism; (2) the route from simple molecules to metabolic network occurred outside vesicles, in free aqueous environment – in a "primitive lagoon" – and only later these molecules become encapsulated inside lipid vesicles. Both the scenarios have some limits and this issue deserves some thought.

If a reaction network arose in solution, how could it then permeate through the double layer? It would imply a high sophistication of the membrane, and it is not reasonable when we talk about primitive conditions. Moreover, there is another considerable difficulty in conceiving the starting point in free solution, since all the molecules would be present in few copies and the probability that they come in mutual relation is low. The letter problem seems to be solved when we introduce to the picture a closed space, like the water pool of a lipid vesicle, in which several molecules could be close enough to interact each other and to give birth to reactions. However, how was it possible that all the molecules indispensable for a metabolism become encapsulated at the same time inside the same compartment?

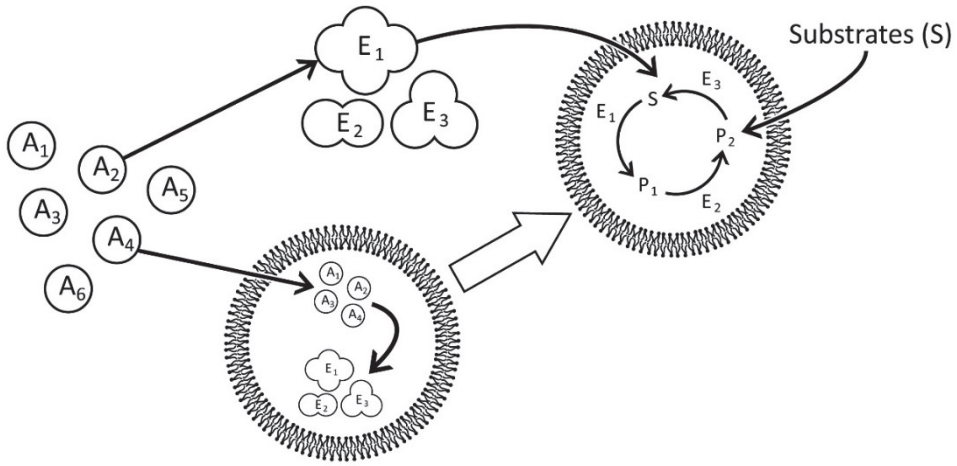

Figure 4.1 The two hypothetical alternatives for the origin of the first metabolism and the protocells, whereby the first proteins and enzymes (E1, E2, E3…) were constructed from free aminoacids (A1, A2, A3, A4…) inside a compartment (bottom), or first outside, then incorporated inside (top). (redrawn after Luisi, 2012).

The term self-organization simplifies the spontaneous set up of the matter into living systems; this process plays a key role in this study and in the whole origin of life vista. Our experimental approach has been designed to test the hypothesis of simultaneous and spontaneous co-entrapment of several molecules, in particular macromolecules, inside lipid vesicles. We formed liposomes by methods which let the lipids self-assemble spontaneously in vesicles, namely the thin film hydration method (on flask surface and on the surface of small glass beads) (Bangham et al., 1965) and the ethanol injection method (Batzri and Korn, 1973). Importantly, we did not use any treatment method which would reduce the heterogeneity of the liposome population or which can induce the passage of macromolecules through the lipid membrane. For example, we avoided to treat the vesicles by the freezing thawing technique, because the resulting offspring would be characterized by homogeneity in terms of solute content (Mayer et al., 1985). We did not extrude the vesicles after their formation for not subjecting them to any physical stress, even if it has been suggested that the extrusion can be compared to the passage of vesicles through the rock pores in primitive times (Hanczyc et al., 2003; Russel, 2003).

We have designed our successful experiments starting from diluted solutions of the molecule of interest (typically from 0.1 to 5 μM) in which the vesicles were formed spontaneously *in situ* and the given samples were observed by confocal microscope. In this simple way, the driving force of the experiment is nothing more than the force of the macromolecules to self-organize (Figure 4.2).

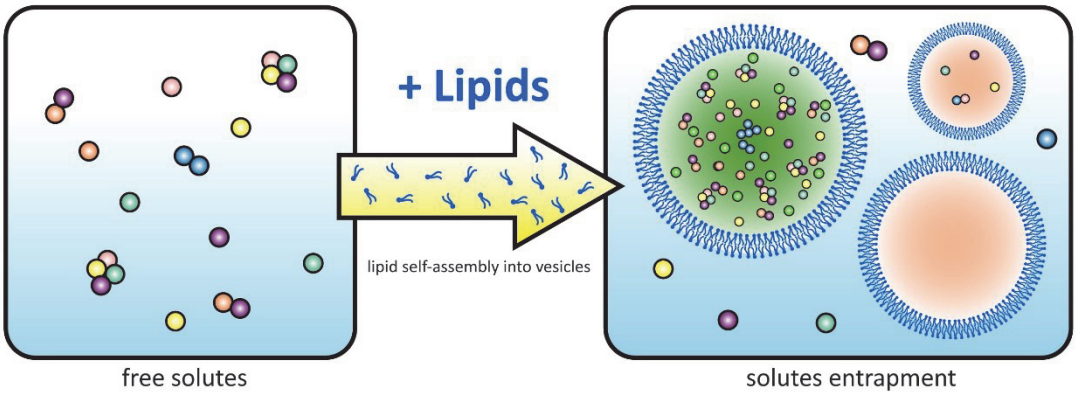

Figure 4.2 Experimental model: spontaneous formation of liposomes in a diluted solution. What results from this method is the emergence of a family of vesicles, which can entrap a big number of macromolecules, overcoming the critical concentration and consequently starting a reaction that in a diluted solution cannot happen.

4.2 Critical threshold concentration

It was already shown (Souza et al., 2009 and 2011) that the formation and the closure of liposomes *in situ* can act as a kind of "attractor" for the free solutes in the aqueous solution, giving a high local concentration. This marvel may overcome the problem of the low efficiency of a reaction when its reactants are diluted in solution. For example, given a certain enzyme concentration $[E]_0$, a simple enzyme/substrate reaction proceeds slowly when the substrate concentration is below the Michaelis-Menten constant, K_m. When the substrate concentration overcome K_m, the reaction rate increases. Moreover, the reaction rate increases linearly with the enzyme concentration. Therefore, if partners of the reaction (enzyme and substrate) have a low concentration, the reaction proceeds sluggishly and it is not very efficient. However, if one of the two reactant, or both, can reach a high local concentration, the reaction rate increases significantly. A similar scenario can be imagined for the formation of complexes. We can speculate that during the formation of big complexes, like ribosomes, a sort of getting-together of primitive nucleic acids and proto-proteins into the same compartment could have helped in the organization of these proto molecules into the first simple ribosomes. The dissociation constant K_d here plays a similar role of the Michaelis-Menten constant. A local concentration threshold must be overcome in order to obtain a high number of complexes otherwise the molecules stay separated and do not acquire the function that emerge when they combine each other.

Our findings may suggest that lipid membrane formation might create a microenvironment able to favour complex formation and to facilitate the onset of a basic metabolism overcoming the problem of the dilution in the external (aqueous bulk) primitive environment. It was shown earlier in our laboratory that there is a mechanism during liposome formation that brings to the formation of super-crowded vesicles. This phenomenon has been clearly illustrated by using different probes like ferritin, ribosomes and proto-ribosomal complexes, together with the use of cryo-transmission electron microscope (Cryo-TEM) (Luisi et al. 2010, de Souza et al., 2011; 2012). The current study confirms and extends these observations to the realm of micrometer-sized vesicles by using fluorescently-tagged macromolecules and laser-scanning confocal microscopy.

With the present results, we first confirmed the presence of a sort of route that brings to the formation of cell-similar liposomes. Moreover, we have also studied dynamic (reactive) biochemical systems that become functioning only after self-concentration inside liposomes. After discovering that some liposomes can entrap enough solutes to reach a solute concentration value to go beyond the K_m of an enzyme/substrate reaction (or beyond K_d for associations), we have also demonstrated that few, but always a significant number of liposomes can include inside their pool more than 80 different molecules in sufficient copies to drive a fluorescent protein synthesis. The success of this study is in the evidence

that the lipid self-organization in membrane may produce a favourable environment for chemical reactions and it could have had a great relevance in the origin of life scenarios.
Our findings are indeed relevant if we consider the formation of the first polymers of life, like proteins or enzyme as well as nucleic acids, as diluted solutions in the warm lagoon. Under this diluted condition, as showed in this work, the fundamental reaction for life could not take place, but when all the components are in a closed space, the reaction can occur efficiently.
From the statistical perspective, we have highlighted that the formation of such “special” solute-filled liposomes is not expected when standard distributions are considered. In fact, for the Gaussian or Poisson distributions the chance to obtain such type of phenomenon is close to zero. This means that special mechanisms, whose driving force can be entropic (like in the hydrophobic effect) might alter the physics of solute capture by vesicles, possibly bringing about solute-crowding in few vesicles, forming cell-like structures that in turn are well capable of efficient internal reactions.

4.3 Speculation on the possible over-crowded vesicles formation mechanism

We do not know yet what mechanism drives this peculiar entrapment, but we have recorded such observations using several probes starting with fluorescent proteins and dextrans with different molecular weights, to observe then a simple enzymatic reaction between carbonic anhydrase (CA) and 6-carboxyfluorescein diacetate (CFDA) giving a membrane impermeable product, the carboxyfluorescein (CF). The most remarkable result was obtained when we extended the study on minimal TX-TL kits (PURE system and *E. coli* extracts from Promega) gaining the same outstanding effect: some liposomes could entrap all the TX-TL components. Therefore, it can be said that the observed phenomenon appears to be general (with respect to macromolecules of different nature, different method of preparation, different vesicle size), keeping constant the lipid structure and the temperature. It is necessary here to highlight the reproducibility of the experiments. If, on one hand, we have found only a small number of super-filled vesicles, generally ~0.1-1% in each sample, on the other hand we can say that this outcome is documented by different studies showing the existence of a mechanism that brings to those structures, and this mechanism is probably due to stochastic events relative to a small number of vesicles.

We exclude that the effect might be a consequence of the electrostatic interaction between solute/lipids, we have chosen a zwitterionic lipid like POPC to reduce these forces. We also made negative controls to see if the macromolecules used could aggregate causing the shifting from the encapsulation of different molecules to the entrapment of a single large agglomerate, confirming the absence of aggregates. In particular, this last consideration has been sufficiently avoided thanks to the experiments performed with a minimal kit composed by 80 macromolecules, all needed for the protein synthesis. The fact that this super- concentration happens only in few vesicles suggests that it is not due to the hypothesis mentioned above, otherwise the vesicles would form all at the same way and the sample would be formed by vesicles containing a similar number of solute molecules.

In the same sample, there is the co-occurrence of “super-filled” vesicle and “empty” vesicles, so we have proposed a model in which empty vesicle are formed as consequence of the fast closure of the open bilayer disc or surface (Lasic, 1988) into a spherical compartment, this rate is considered close to ~1 ms^{-1} (Hernàndez-Zapata et al., 2009). The formation of a super-filled liposome may be triggered by a slow lipid disc closure mechanism thanks to temporary interaction between some solute molecules and the lipid disc before the vesicle closure. This establishes a cooperative mechanism that let a slow vesicle closure, and a consequent solute incoming and formation of a super-crowed liposome.

The possible two mechanisms behind the heterogeneity of the vesicles is displayed in Figure 4.3.

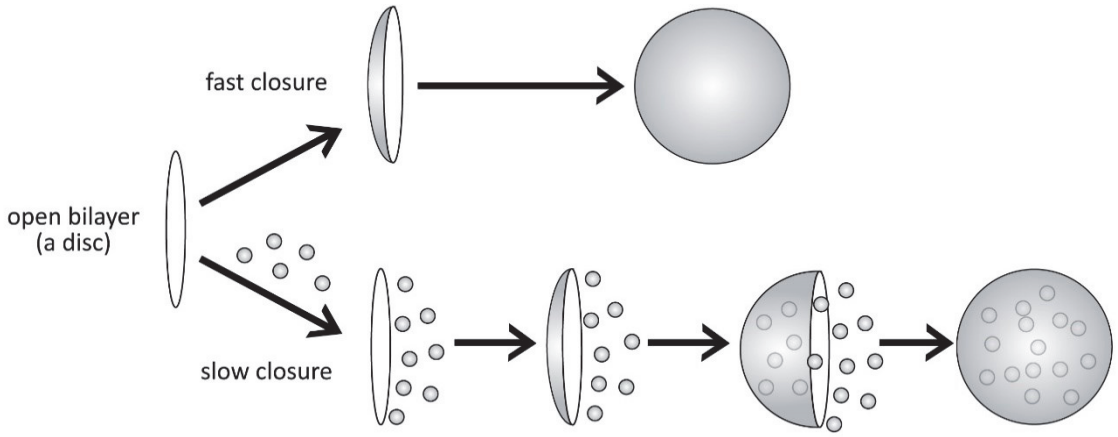

Figure 4.3 A hypothesis about the mechanism, based on kinetic bilayer closure, for the formation of empty and solute-filled vesicles. Starting from an open bilayer (discs—open surface in three dimensions) there may be two different mechanisms that are represented by unstable intermediate structures derived from lipid self-assembly. These bilayer surfaces, after reaching a sufficient size, can close on themselves and form vesicles (Lasic 1988). We assume that the fast closure of the discs/surfaces brings about empty vesicles, and this is the dominant process within the vesicle population. In contrast, possibly caused by stochastic and non-specific solute-bilayer interaction (which transiently ‘‘stabilizes’’ the disc/surface), a slow closure rate could allow further molecules to be entrapped. (Redrawn after Souza et al., 2011)

4.4 Why is macromolecular crowding important?

Natural cells are characterized by high total concentration of molecules in their interiors, such media are called “crowded” rather than “concentrated” because no single macromolecular species occurs at high concentration but, taken together, the macromolecules occupy a significant portion (typically between 10% and 40%) of the total volume (Fulton, 1982; Record et al., 1998; Ellis, 2001). Consequentially in nature most macromolecular reactions are carried out in crowded environment developed by the cell itself and that crowding can alter the reactivity of individual macromolecules (quantitatively and qualitatively).

The fact that the cell shows this feature would be a sufficient motivation to investigate deeply this phenomenon also making synthetic models. Nevertheless, macromolecular properties are generally studied in dilute solution, in which the total macromolecular concentration is around 1-10 g l^{-1} and those values are very far from the actual concentration *in vivo*, considering, for example, that the total concentration of proteins and RNA inside a cell of Escherichia coli is circa 300-400 g l^{-1} (Zimmerman and Minton, 1991). Biological macromolecules have developed and evolved in such crowded environment, so there might be an advantage in this kind of structure.

The macromolecular crowding has chemical consequences, for example the macromolecular compaction and association (Minton 2000; 2001; Ellis 2001) that are phenomena which lead to a decrease in excluded volume. Obviously, the crowding will affect the association of macromolecules that have tendency to associate, it will not create this tendency *de novo* (Rivas et al, 2004).

It has been seen that this phenomenon has effects for genome structure and function because it influences the structural organization of DNA and the interaction between DNA and proteins (Zimmerman, 1993; Zimmerman and Murphy, 1996). From the Origin of life viewpoint, particularly the origin of the first oligomers, it is also remarkable the study showing that enzymes could synthetize polypeptides when they are under crowded condition, in contrast to their tendency to catalyse peptide hydrolysis in dilute solution (Somalinga and Roy, 2002).

So crowding can dramatically influence biochemical kinetics by volume exclusion effects that reduce diffusion rates and enhance binding rate of macromolecules, and recently has been demonstrated that it increases the robustness of gene expression through integrating synthetic cellular components of biological circuits and artificial cellular nanosysthems (Tan et al, 2013).

The finding that there is a phenomenon that lets few vesicles incorporate spontaneously a big number of macromolecules can help in different ways, for example, once we know the mechanism that leads to this kind of vesicles it will be possible develop a vesicle formation

method in order to maximize the formation of this particular crowded vesicles. It will be useful for applications that use realistic cell-models in biochemistry, biophysics and cell biology.
Regarding the origin of life, we could investigate whether the crowding phenomenon might have facilitated the establishment of a prebiotic metabolism, not only because all the components of such metabolism are in the same vesicle, but also because it could help in the binding between macromolecules.

4.5 Contribution of this study

In this PhD thesis, we report some new evidences contributing to the ongoing study of the origin of protocells, in particular addressing the question on how multiple macromolecular solutes could be captured within a lipid vesicle and later give rise to proto-metabolism. We have documented the spontaneous formation of few vesicles (within a population), which could entrap a huge number of different macromolecules in their water pool against the statistical prediction. We have seen indeed the formation of a heterogeneous family of vesicles, the majority were empty and few of them were super filled, showing an “all-or-nothing” trend (Luisi, 2012).
In some cases we obtained vesicles with a crowding index similar to biological cells, and this is a remarkable observation, since it is known that in these conditions the molecular reactivity is enhanced (Zhou et al., 2008) because the volume of water molecules is reduced to give space to the solutes. This discovery imposes a revision of current knowledge on the emergence of protocells in primitive (diluted) solutions. In particular, the problem of the needed high local concentration of reactants inside protocells and the problem of few molecules in solution seem to be alleviated in great extent thanks to these new self-organization pathways. In fact, the vesicle closure mechanism could have played an unexpected key role in the origin of life. We can say that the lipid membrane does not work only as a protective shell, but it had an active part in the origin of the first living entities. The vesicle lumen becomes, then, thanks to this high local concentration, a favourable microenvironment suitable for the chemical and biological reactions.
In this study is shown how the components of a simple enzymatic reaction first, and an entire set of molecules for protein formation then, can be incorporated so efficiently in some liposomes. In this way, it was possible to reach a critical concentration in order to start and to carry out the reaction, which could work more efficiently in the closed compartments than in the bulk solution. We think that this experimental model address the question of the origin of prebiotic metabolism, bypassing the problem of the limited permeability of the membranes and the problem to reach a given solute concentration.

Simple and complex molecular systems (from individual enzymes to transcription-translation kits) share the similar pattern, favouring the crowding of these structural units inside vesicles.

4.6 Open questions and perspective

Our investigation on the “super-crowded vesicles” is still in progress despite the new discoveries here discussed, in fact, there are several gaps to fill and most of them are linked to the mechanistic details of these new brand structures formation.

If this topic is attracting from the perspective of the origin of life, we have also to say that it could have relevance in biotechnology, in the applications based on liposomes as drug delivery systems or in future technologies centred on synthetic cells that need a high reaction efficiency. Once understood the forces that let this phenomenon happen, we can try to develop standard liposomes preparation methods in order to make a large number of super-filled vesicles. For this purpose it would be necessary extend the study to different kind of molecules starting from the membrane compounds, we could use other lipids to have a panoramic view on the role played by the chemical structures on the self-filling mechanism displayed by some vesicles. Furthermore, for primitive chemistry it is significant study the particular case of fatty acids-vesicles, the fatty acids are indeed considered the building blocks of the primordial membranes and see if we can obtain the same results achieved with phospholipids can support our theory on the role of the membrane in the earliest stages of life.

Even if a wide variety of probes have been used in this project and in the preliminary ones, it would be indispensable extend it to further molecules in order to see if, for example, the same principle works also with low-molecular weight to arrive to larger molecules like nucleic acids. Can the length of the molecules affect the result? To answer to this question we have already started experiments involving polyethylene glycols (PEGs) with very different molecular weights, these tries will be amplified using DNA and RNA.

At the end, the most attractive model would be the formation of fatty acids vesicles in a diluted solution of nucleic acids, preliminary data have shown that the atypical concentration can be observed also in this case (manuscript in preparation).

Previous studies were focused on nm-sized vesicles and also the present ones are relatively small vesicles (ca.1-2 μm), can we see the crowding effect also inside giant vesicles (GVs)?

5 Summary

One of the most ambitious goals of synthetic biology is to construct, following a bottom-up approach, cell-like systems of minimal complexity for understanding fundamental aspects of biology – as the origin of life problem, for example – and for potential application in biotechnology. The experimental approaches to the construction of *minimal cells* play a key role in understanding how primitive cells originated from inanimate matter. The scientific community agrees that in primitive time the inorganic matter underwent a kind of slow and spontaneous "chemical evolution" that, powered by self-organization, lead to the formation of the first cells. From those very simple and rudimentary structures, all living organisms resulted after billions of years of evolution.

In our research group, we focus on understanding how primitive cells originated from non-living components (single molecules) by combining liposome technology and biomolecular systems. This will generate knowledge for the *minimal cell* project and, at the same time, will allow us discovering possible routes that lead, more than 3.5 billions years ago, to cellular systems.

In this particular project, we have investigated the origin of primitive metabolism-supporting cells via the construction of experimental models. We think that the lipid membrane played an essential role for setting up systems with realistic metabolic potentiality at the very early stage. By a careful study of lipid vesicle populations, prepared in the presence of several types of solutes (proteins, nucleic acids), we have reported the coexistence of empty and *super-filled* lipid vesicles. The large number of experiments and the many different tested conditions strongly suggest that our observations are general and correspond to a spontaneous pathway for concentrating solute molecules inside (a small number of) vesicles. The lipid shell, then, has not any longer the single function of protection, but it plays an active role recruiting molecules in the vesicle lumen, so that critical concentration thresholds to trigger biochemical reactions in a microenvironment are easily reached. We could show that a simple enzymatic reaction is facilitated inside a liposome water pool as opposed to bulk or other vesicles. We have also conducted experiments by forming liposomes in a diluted solution of a protein synthesis minimal kit. We have observed that the reaction did not work efficiently in bulk due to the macromolecules' dilution; however, a certain number of liposomes could efficiently entrap all 80 macromolecules needed for the desired reaction in a sufficient number of copies.

Our results indicate that unsolved problems in origin of life scenarios, like the issue of low solute concentration and the low membrane permeability, could have been solved by the reported spontaneous entrapment and self-concentration of molecules in cell-like particles.

Zusammenfassung

Eins der ambitioniertesten Ziele der synthetischen Biologie ist es, mittels eines *Bottom Up*-Ansatzes zelluläre Strukturen minimaler Komplexität zu erzeugen, um grundlegende Aspekte der Biologie – wie zum Beispiel die Frage nach dem Ursprung des Lebens – zu erforschen oder potentielle Anwendungen in der Biotechnologie zu entwickeln.

Die experimentelle Annäherung an die Konstruktion dieser *Minimal-Zellen* spielt eine zentrale Rolle für das Verständnis der Bildung ursprünglicher Zellen aus unbelebter Materie. Die wissenschaftliche Gemeinschaft ist sich einig, dass anorganische Materie in der Frühzeit der Erdgeschichte eine Art langsamer und spontaner „chemischer Evolution" durchlief, die – von Selbstorganisation angetrieben – zur Bildung der ersten Zellen geführt hat. Ausgehend von diesen einfachen und rudimentären Strukturen haben sich über Milliarden von Jahren der Evolution alle lebenden Organismen entwickelt.

Unsere Arbeitsgruppe befasst sich mit dem Verständnis primitiver Zellen aus unbelebter Materie (Einzelmolekülen) auf Basis einer Kombination von liposomaler Technologie und biomolekularen Systemen. Auf diese Weise lassen sich nicht nur Erkenntnisse für das m*inimal-cell*-Projekt gewinnen, gleichzeitig erlaubt dieser Ansatz die Entdeckung möglicher Wege, die vor über 3.5 Milliarden Jahren zur Bildung zellulärer Systeme geführt haben könnten.

Im Rahmen des vorliegenden Projektes haben wir den Ursprung primitiver metabolisierender Zellen über die Konstruktion experimenteller Modelle untersucht. Wir denken, dass Lipid-Membranen eine entscheidende Rolle dafür gespielt haben, dass bereits in einer frühen Phase Systeme mit nachweisbaren metabolischen Fähigkeiten ausgebildet wurden. Durch intensive Studien an Lipid-Vesikel-Populationen, die in Gegenwart verschiedener Grundbausteine (Proteine, Nukleinsäuren) gebildet wurden, konnten wir zeigen, dass leere und *über-volle* Vesikel nebeneinander existieren. Die Vielzahl an Experimenten unter verschiedenen Testbedingungen legen nahe, dass unsere Beobachtungen allgemein gültig sind und einen Weg aufzeigen, der die spontane Konzentration gelöster Moleküle innerhalb von (wenigen) Vesikeln ermöglicht. Die Lipid-Hülle erfüllt damit nicht länger nur eine Schutzfunktion, sie trägt aktiv dazu bei, Moleküle im Vesikelinnenraum anzusammeln, so dass kritische Grenzkonzentrationen für den Start biochemischer Reaktionen innerhalb dieser Mikro-Umgebung erreicht werden können. Wir konnten zeigen, dass eine einfache enzymatische Reaktion in einer Liposom-Wasser-Dispersion im Vergleich zu einfachen Lösungen oder verschiedenen anderen Vesikeln verbessert abläuft. Weiterhin haben wir Experimente durchgeführt, in denen wir Liposomen mit einem *„minimal kit"* zur Proteinsynthese in verdünnter Lösung versetzt haben. Dort konnten wir beobachten, dass die Reaktion in Lösung aufgrund der hohen Verdünnung der beteiligten Makromoleküle nicht gelang, während eine bestimmte Anzahl von Liposomen

in der Lage war, alle 80 für die gewünschte Reaktion benötigen Makromoleküle in ausreichender Anzahl einzuschließen, obwohl die Zufallsstatistik entschieden dagegen spricht.

Unsere Ergebnisse zeigen, dass bisher ungelöste Probleme der Forschung zum Ursprung des Lebens, wie zum Beispiel die geringe Konzentration benötigter Bausteine in Lösung oder die geringe Permeabilität von Membranen, durch die vorliegenden Ergebnisse zu spontanem Einschluss und dadurch erhöhter Konzentration von Molekülen in zellähnlichen Partikeln beantwortet werden können.

6 References

Alberts B., Wilson J., Hunt T. (2008). Molecular biology of the cell. *New York : Garland Science.*

Bachmann P.A., Luisi P.L. and Lang J. (1992). Autocatalytic self-replication of micelles as models for prebiotic structures. *Nature*, **357**, 57-59.

Bachmann P.A., Walde P., Luisi P.L., Lang J. (1990). Self-Replicating Reverse Micelles and Chemical Autopoiesis. *J. Am. Chem. Soc*., **112**, 8200-8201.

Bangham A.D., Standish M.M., Watkins J.C. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Bio*., **13**, 238-252.

Batzri S. and Korn E.D. (1973). Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Acta*, **298**,1015-1019.

Bloechliger E., Blocher M., Walde P. and Luisi P.L. (1998). Matrix effect in the size distribution of fatty acids vesicles*. J. phys. Chem*. **102**, 10383-10390.

Britt R.R. (2000). Are we all aliens? The new case for panspermia. In *http://www.space.com*.

Chen I.A., Roberts R.W., Szostak J.W. (2004). The emergence of competition between model protocells. *Science*, **305**, 1474-1476.

Darwin C. (1859). On the origin of Species by Means of Natural selection. *John Murray, London*

Deamer D.W. (1998). Membrane compartments in prebiotic evolution; in: A. Brack (Ed.) The molecular origins of life: assembling pieces of the puzzle. *Cambridge University Press, Cambridge,* 189-205.

Deamer D.W. (1985). Boundary structures are formed by organic components of the Murchison carbonaceous chondrite. *Nature,* **317**, 792-794.

De Duve C. (1991*).* Blueprint for a Cell: The Nature and the Origin of Life*. Neil Patterson Publishers.*

De Lorenzo V. and Danchin A. (2008). Synthetic biology: discovering new worlds and new words. *EMBO Rep*. 9(**9**): 822–827.

Ellis RJ (2001) Macromolecular crowding: obvious but underappreciated. *Trends Biochem Sci* **26**: 597–604

Eschenmoser A. and Kisakürek M.V. (1996). Chemistry and the origin of life. *Helv. Chim. Acta,* **79**, 1249-59.

Fulton A.B. (1982) How crowded is the cytoplasm? *Cell* **30**: 345–347

Gibson D.G., Glass J.I.; Lartigue C., Noskov V.N., Chuang R.Y., Algire M.A., Benders G.A., Montague M.G., Ma L., Moodie M.M., Merryman C., Vashee S., Krishnakumar R., Assad-Garcia N., Andrews-Pfannkoch C., Denisova E.A., Young L., Qi Z., Segall-Shapiro T.H., Calvey C.H., Parmar P.P., Hutchison III C.A., Smith H.O., Venter J.C. (2010). Creation of a Bacterial Cell Controlled by a Chemically Synthesized Genome. *Science*, **329**, 52-56.

Gil, R., Silva, F.J., Peretó, J., e Moya, A. (2004). Determination of the core of a minimal bacteria gene set. Microb. Molec. Biol. Rev., **68**, 518-37.

Haldane J.B.S. (1929). The origin of life. *Rationalist Annual*, **148**, 3-10.

Haldane J.B.S. (1954). The origin of life*. New Biol*., **16**, 12-27.

Haldane J.B.S. (1967). In The Origin of Life, edited by *J.D. Bernal. World Publishing Co.*

Halliday A.N. (2000). Terrestrial accretion rates and the origin of the Moon. *Earth Planet Sci.* Lett.,**176**, 17-30.

Hanczyc M.M., Fujikawa S.M., Szostak J.W. (2003). Experimental models of primitive cellular compartments: Encapsulation, growth, and division. *Science* **302**: 618–622.

Hernández-Zapata E., Martínez-Balbuena L., Santamaría-Holek I (2009). Thermodynamics and dynamics of the formation of spherical lipid vesicles. *J Biol Phys* **35**: 297-308.

Hoyle F. and Wickramasinghe C. (1999). Astronomical origins of life – Steps toward panspermia. *Astrophys. Space Sci*., **268**, VII-VIII.

Kita H., Matsuura T., Sunami T., Hosoda K., Ichihashi N., Tsukada K., Urabel and Yomo. (2008). Replication of Genetic Information with Self-Encoded Replicase in Liposomes. *Chem. Bio. Chem*., **9**, 2403-10.

Kurihara K., Tamura M., Shohda K., Toyota T., Suzuki K., Sugawara T. (2011). Self-reproduction of supramolecular giant vesicles combined with the amplification of encapsulated DNA*. Nat Chem*. **3**, 775-778.

Lasic D.D. (1988). The mechanism of vesicle formation. *Biochem J*, **256**, 1-11

Lazcano A. and Miller S.L. (1996). The origin and early evolution of life: prebiotic chemistry, the pre-RNA world and time*. Cell Press* **85**, 793-708.

Luisi P. L. (2012). An open question on the origin of life: the first forms of metabolism. *Chemistry & Biodiversity*, **9**, 2635-2647.

Luisi P.L., Ferri F. and Stano P. (2006). Approaches to semi-synthetic minimal cells: review. *Naturwissenschaften*, **93**, 1-13.

Luisi P.L., Oberholzer T., e Lazcano A. (2002). The notion of a DNA minimal cell: a general discourse and some guidelines for an experimental approach. *Helv. Chim. Acta*, **85** (6), 1759-77.

Luisi P.L., Vonmont- Bachmann P., Fresta M. (1993). Self-Reproduction of Micelles and Liposomes and the Transition to Life. *Journal of Liposome Research*, **3**, 63l-638.

Luisi P.L., e Varela F.J. (1990). Self-replicating micelles – a chemical version of minimal autopoietic systems. *Orig. Life Evol. Biosph*., **19**, 633-43.

Maturana H. and Varela F. (1980). Autopoiesis and Cognition: The Realization of the Living. *Reidel*.

Mayer L.D., Hope M.J., Cullis P.R., Janoff A:S: (1985). Solute distributions and entrapping efficiencies observed in freeze-thawed multilamellar vesicles. *Biochim. Biophys. Acta*, **817**, 193-196.

Miller S.L. (1953). A production of amino acids under possible primitive Earth conditions. *Science* **117**, 2351-61.

Minton AP (2000) Implications of macromolecular crowding for protein assembly. *Curr Opin Struct Biol* **10**: 34–39

Mojzsis S.J., Harrison T.M and Pidgeon R.T. (2001). Oxygen-isotope evidence from ancient zircons for liquid water at Earth's surface 4,300 Myr ago. *Nature* **409**, 178-181.

Morowitz, H.J. (1992). Beginnings of Cellular Life. *Yale University Press*.

Noireau, V., Bar-Ziv R. and Libchaber A. (2003). Principles of cell-free genetic circuit assembly. *Proc. Natl. Acad. Sci*. USA, **100**, 12672-77.

Oberholzer T., Nierhaus K.H., and Luisi P.L. (1999). Protein expression in liposomes. *Biochem. Biophys. Res. Commun*., **261**, 238-41.

Oberholzer T., Albrizio M. and Luisi, P.L. (1995a). Polymerase chain reaction in liposomes. *Curr. Biol*., **2**, 677-82.

Oberholzer T., Wick R., Luisi P.L., and Biebricher C.K. (1995b). Enzymatic RNA replication in self-reproducing vesicles: an approach to a minimal cell. *Biochem. Biophys. Res. Commun.*, **207**, 250-57.

Oparin, A.I. (1957). The Origin of Life on Earth, III Ed., London. *Academic Press*.

Oparin, A.I. (1953). The Origin of Life. New York. *Dover Publications*.

Oparin, A.I. (1924). Proishkhozhddenie Zhisni. Mosca. *Moskowski Rabocii*.

Oró, J. (1960). Synthesis of adenine from ammonium cyanide. *Biochem. Bioph. Res. Commun.*, **2**, 407-12.

Pietrini A. V., Luisi P. L. (2004). Cell-free Protein Synthesis through Solubilised Exchange in Water/Oil Emulsion Compartments. *ChemBioChem*, **5**, 1055-1062.

Powner M.P., Gerland B., e Sutherland J.D. (2009). Synthesis of activated pyrimidine ribonucleotides in prebiotically plausible conditions. *Nature*, **459**,239-242.

Record TM Jr, Courtenay ES, Cayley S, Guttman HJ (1998) Biophysical compensation mechanisms buffering E. coli protein–nucleic acid interactions against changing environments. *Trends Biochem Sci* **23**: 190–194

Rivas G., Ferrone F. and Herzfeld J. (2004). Life in a crowded world. *EMBO reports* **5**, 23–27.

Russell M.J. (2003). The importance of being alkaline. *Science*, **302**(5645), pp. 580-581.

Schopf J.W. (1998). In The Molecular Origin of Life, edited by Brack A. *Cambridge University Press*.

Schopf J.W. (1993). Microfossils of the early archean apex chert: new evidence of the antiquity of life. *Science*, **260**, 640-46.

Schopf J.W. (1992). In The Proterozoic Atmosphere, edited by J.W. Schopf and C.Klein. *Cambridge University Press*.

Shostak S. (2003). Panspermia: Spreading Life Through the Universe. *The SETIInstitute*.

Soga H, Fujii S, Yomo T, Kato Y, Watanabe H, Matsuura T. (2014) In vitro membrane protein synthesis inside cell-sized vesicles reveals the dependence of membrane protein integration on vesicle volume. *ACS Synth Biol*, **3**, 372-379

Somalinga B., Roy R. (2002). Volume exclusion effect as a driving force for reverse proteolysis. *J Biol Chem* **277**: 43253–43261

Souza, T.; Stano, P.; Steiniger, F.; D'Aguanno, E.; Altamura, E.; Fahr, A.; Luisi, P. L. (2012). Encapsulation of Ferritin, Ribosomes, and Ribo-Peptidic Complexes Inside Liposomes: Insights Into the Origin of Metabolism. *Orig. Life Evol. Biosph.*, **42**, 421-428.

Souza, T.P., Stano, P., e Luisi, P.L. (2009). The minimal size of liposome-based model cells brings about a remarkably enhanced biological activity. *Chem. Bio. Chem*, **10**, 1056-1063.

Souza T., Steiniger F., Stano P., Fahr A., Luisi P. L. (2011). Spontaneous crowding of ribosomes and proteins inside vesicles: A possible mechanism for the origin of cell metabolism. *ChemBioChem*, **12**, 2325-2330.

Stano, P.; Carrara, P.; Kuruma, Y.; Souza, T.; Luisi, P. L. (2011). Compartmentalized reactions as a case of soft-matter biotechnology: Synthesis of proteins and nucleic acids inside lipid vesicles. *Journal of Material Chemistry*, **21**, 18887-18902.

Stano P. and Luisi P.L. (2010). Achievements and open questions in the self-reproduction of vesicles and synthetic minimal cells. *ChemComm*, **46**, 3639-3653.

Szostak, J.W., Bartel, D.P., e Luisi P.L. (2001). Synthesizing life. *Nature*, **409**, 387-90.

Tan C., Saurabh S., Bruchez M., Schwartz R. and LeDuc P (2013). Molecular crowding shapes gene expression in syntheticcellular nanosystems. *Nat Nanotechnol*. 8(8): 602–608

Torre P., Keating C.D., Mansy S.S. (2014). Multiphase water-in-oil emulsion droplets for cell-free transcription-translation. *Langmuir*. **30**, 5695-5699.

Varela F.J., Maturana H.R., and Uribe R.B. (1974). Autopoiesis: the organization of living system, its characterization and a model. *Biosystems*, **5**, 187-96.

Walde P., Wick R., Fresta M., Mangone A., Luisi P.L. (1994).Autopoietic Self-Reproduction of Fatty Acid Vesicles. *JACS* **116**, 11649-11654.

Wilde S.A., Valley J.W., Peck W.H. and Graham C.M. (2001). Evidence from detrital zircons for the existence of continental crust and oceans on the Earth. *Nature* **409**, 175-178.

Yu W., Sato K., Wakabayashi M., et al. (2001). Synthesis of functional protein in liposome. J. *Biosc. Bioeng*., **92**, 590-93.

Zhou H-X, Rivas G., Minton A.P. (2008). Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. *Annu. Rev. biophys*., 37: 375-397.

Zimmerman SB, Murphy LD (1996). Macromolecular crowding and the mandatory condensation of DNA in bacteria. *FEBS* Lett **390**: 245–248

Zimmerman SB (1993). Macromolecular crowding effects on macromolecular interactions: some implications for genome structure and function. *Biochim Biophys Acta* 1216: 175–185

Zimmerman SB, Minton AP (1993) Macromolecular crowding: biophysical, biochemical, and physiological consequences. *Annu Rev Biophys Biomol Struct* **22**: 27–65

7 Abbreviations

A	Amino acid
C.a.c .	Critical aggregation concentration
CA	Carbonic anhydrase
CF	Carboxyfluorescein
CFDA	6-Carboxyfluorescein diacetate
E	Enzyme
GFP	Green fluorescent protein
GV	Giant vesicles
*K*d	Dissociation constant
*K*m	Michaelis-Menten constant
LUCA	Last Universal Common Ancestor
PEG	Polyethylene glycols
POPC	1- palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine
Rib 1	Ribozime 1
Rib 2	Ribozime 2
SSMC	Semi-Synthetic Minimal Cell
TX-TL	Transcription-translation

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Statement

I am very familiar with the Promotionsordnung of the Faculty of Biology and Pharmacy in the Friedrich-Schiller-University Jena. I produced the dissertation by myself. Hereby I declare that this dissertation does not contain any material previously submitted for a degree or diploma at another university or any material previously written or published by any other person, except where due acknowledgement or reference has been made in the text. I also declare that I did not enlist the assistance of any dissertation-counselling agent, and that I did not provide any direct or indirect monetary benefit to any third party for work connected to my dissertation.

Jena, 10.09.2015

Erica D'Aguanno